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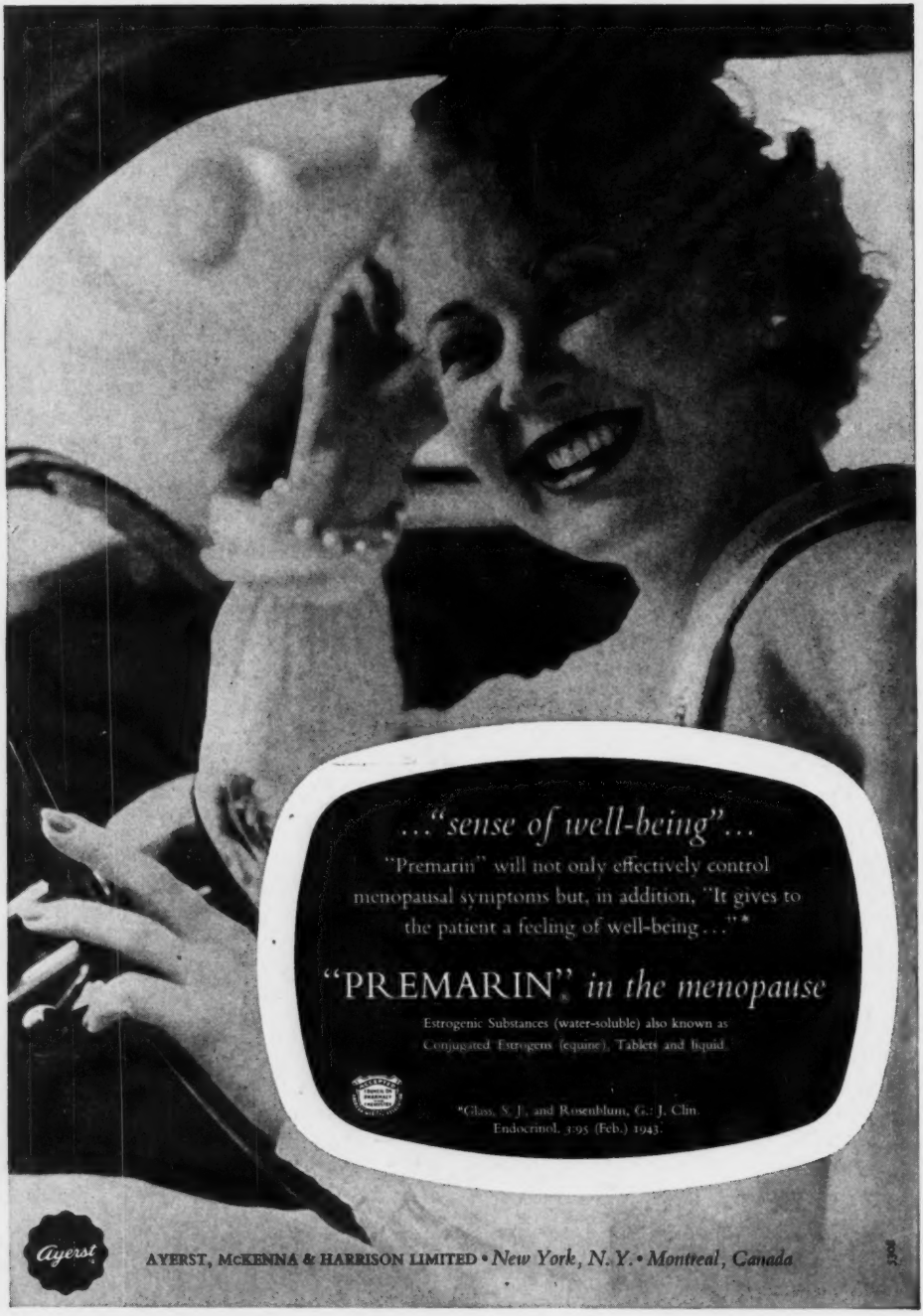
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A BIO-ASSAY METHOD FOR THE EVALUATION OF ADRENOCORTICAL POTENCY BY MEANS OF EXPERIMENTAL TRAUMA, WITH SHOCK, IN RATS*

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AMONG the various current methods of adrenocortical bio-assay, the "stress-survival" type has been recognized for some time (Dorfman, 1949). This approach makes use of the adrenalectomized animal which is subjected to some form of stress after having been pre-treated with measured amounts of a substance known to possess adrenocortical activity (the so-called "reference standard"), to be compared with similar animals pre-treated with the material to be tested. The relative degree of protection against death, expressed in terms of percentage survival, is used to compute the potency of the test-material based upon the responses observed in the reference-standard groups.

This approach was used in developing the method of bio-assay as described below. Stress is induced by means of a device known as the "Noble-Collip Drum" whereby it is possible to produce experimental trauma in a qualitatively and quantitatively reproducible manner in small animals. Such trauma, if sufficiently extensive, can be made to result in death shown to be due to the effects of traumatic shock. The apparatus was described by Noble and Collip (1942). It consists of vertically mounted drums fitted with narrow shelves which project from the inner periphery at an angle such that upon rotation, the animal inside is carried upwards to the point where he falls off striking the side of the drum. The successive impacts of these falls constitute the total stress which can be controlled as to degree by regulating the total number of drum revolutions and the number of projecting shelves. The advantage of this procedure over others lies in the elimination of extraneous factors such as anesthetization, fractures, cerebral lesions, and

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free hemorrhage (especially into the peritoneal cavity from hepatic and/or splenic rupture) any of which were found to interfere with consistently reproducible mortality rates from one experiment to another.

The concept of applying this technique towards a method of adrenocortical bio-assay arose from some of the findings of these same authors showing that by adequate pretreatment with adrenocortical extract, adrenalectomized rats were able to survive drum-stress as well as (and in some instances, better than) intact animals. Experiments were thus undertaken to establish a standardized procedure whereby to test for a possible linearity in dosage-response in pre-treated adrenalectomized rats.

Apparatus: In Figure 1 is shown the modified Noble-Collip drum assembly used in this work. The four-drum unit consists of circular metal drums (A) 15″ in diameter and 8″ deep. The covers (B) are of *Plexiglass* and allow for the visual observation of the animal at all times. The opening (C) in the center facilitates both introduction and removal of test animals as well as individual examination by palpation without stopping the drums. Two shelves (D) are located diametrically opposite inside the drum, fixed at an angle of about 45° to the periphery. Under the standardized velocity used, these shelves cause the animal to fall through a distance of about 8″ twice per revolution. All four drums are rotated simultaneously and uniformly by a motor (F) whose speed can be regulated as desired. The total number of drum-revolutions is automatically recorded by a suitable counting device.

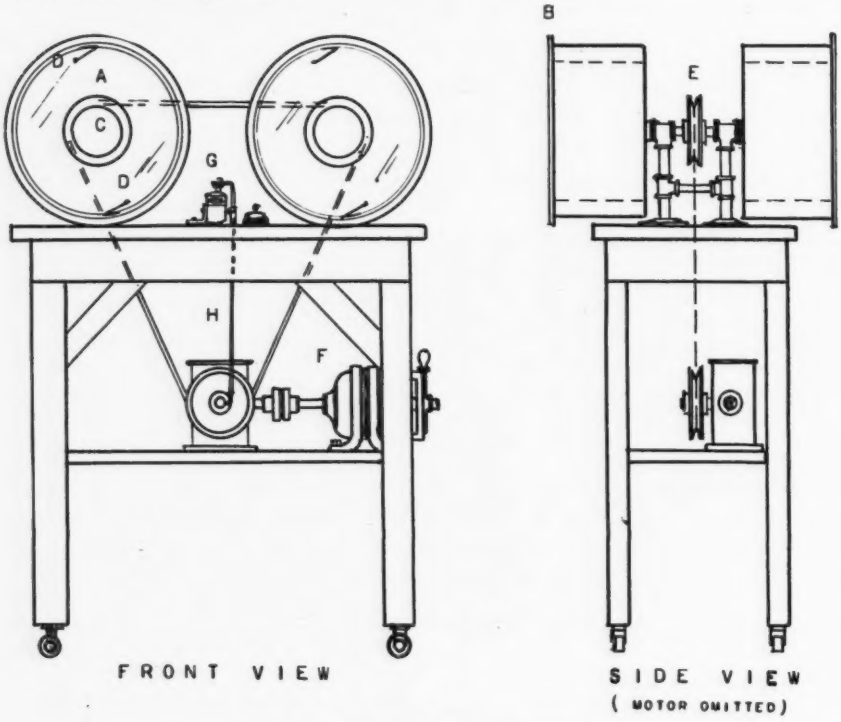

FIG. 1. Rotating drum assembly.

Test animals: Male albino (Charles River Strain) 6–7 week-old rats, weighing 140–170 grams at adrenalectomy, and 125–150 grams at the time of testing were used throughout. This range of age and weight was found optimal for consistent responses. The animals were obtained from the colony of a commercial firm (The Charles River Breeding Laboratories, Boston, Mass.), transported to the hospital animal room where they were housed in regular cages and fed their usual diet with tap water to drink. Efforts were made insofar as possible to maintain the same nutritional and environmental conditions under which the animals had been reared. Their food was therefore purchased from the same firm that supplied the animals. Room temperature was kept, within reasonable limits, the same (about 78° F.) as that of the supplier's animal room. No attempt was made to simulate relative humidity. To ensure further uniformity, an interval of not less than three days' duration was allowed for adequate adaptation before using the rats for experimentation.

Adrenalectomy and postoperative care: After adaptation, the rats were adrenalectomized under USP Anesthesia ether. The duration of initial anesthetization within the chamber was about one minute with the concentration of ether vapour kept as constant as possible throughout. Immediately after operation the rats were returned to their cages where they were maintained for 24 hours on their usual diet with 0.9% saline to drink. They were then transferred to cages with single compartments for the subsequent 24 hours without food or saline, but with tap water offered *ad lib.*

Preparation and administration of test-material: All substances tested were first dissolved in absolute (CP) ethanol at concentrations of 5–10 mg./ml. These stock solutions were then diluted prior to injection so that the required dosage per rat was dissolved in 1 ml. of 20% aqueous ethanol. Injections were given subcutaneously at 2-hour intervals in three stages: one half the total dose initially, followed by two equally divided doses of the remainder. The "zero-control" animals were identically injected with 20% aqueous ethanol.

Method of inducing drum-stress: In this method, the consistency of responses from test to test depends largely upon two factors: a) drum velocity (i.e., the *rate* at which stress is inflicted), and b) the total number of drum revolutions (i.e., the *degree* and *duration* of the stress). Optimal consistency was found to occur under our experimental conditions when the drums were rotated at 36–37 RPM for a total of 234 revolutions, the rats' fore- and hind-limbs being taped approximately ½" apart with 1" adhesive tape (the unfettered animal is able to jump across the projections and can thus vary the total number of impacts sustained).

The standardized procedure is as follows: Two hours after the final injection, a group of 4 rats, 2 controls and 2 injected with test-material, is introduced into the respective drums and run simultaneously for 234 turns. Immediately thereafter the binding tape is severed and the animals returned to their cage compartments. The time o'clock at the end of the run is recorded and the animals observed thereafter in order to record the time of death. Successive groups of 4 rats are then run until all the animals in the test have been stressed. After removal from the drums the animals are handled as little as possible up to the point where respiration has apparently ceased. The animal is then examined further and is considered dead when palpable heart beat and respiratory movements are no longer detectable. In this way the time of death can be ascertained with reasonable consistency within 3 or 4 minutes at most.

Postmortem examinations: In all cases autopsies were done as soon after death as possible. Completeness of adrenalectomy was always checked and animals showing obvious adrenal remnants were discarded. Signs and symptoms of traumatic shock were like-

wise sought. These constituted a marked engorgement of the inferior vena cava and of the right heart with dark, viscous blood; similar engorgement of the liver and spleen with gross enlargement of the latter; very pronounced hemorrhagic lesions within the lumen of the distal two-thirds of the small intestine; and marked dilatation of the caecum and its blood vessels, the latter being engorged to the extent that the entire caecum appeared purplish in colour. This picture was invariably seen in animals dying within 18 hours after drum stress but was always absent in those surviving 18 hours or longer.

Dosage-response to compound "F" and Statistical Analysis: Kendall's compound "F" (11,17-hydroxycorticosterone, m.p. 208–214°) was selected as the reference standard in the initial experiments testing for linearity in dosage-response in drum stressed rats.

As previously mentioned, the time interval between cessation of drum stress and the death of the animal was consistently recorded. These various after-stress survival times were eventually segregated so as to fall within six arbitrarily chosen end-points, viz., 15, 20, 30, 60 and 120 minutes, and 18 hours. The percentage of survivors in each of the variously treated groups was then computed for each of these intervals.

The mean values of percentage survival responses in rats treated with compound "F" are shown in Table 1 which also includes similar data for untreated, sham-adrenalectomized animals. In Figure 2 these values are presented graphically and the degree of protection afforded by the increasing dosages of compound "F" is obvious.

In seeking to discover which of these end points yields optimal information, analysis of variance was undertaken for the separate values of the responses at each end point observed employing the equation: $R=a+bx$, where $R=\%$ survival at any end point; $a=$intercept; $b=$slope; and $x=\log$ (dosage+1). (Dosage+1 is used to enable the inclusion of the "zero-dosage" observations.)

TABLE 1. MEAN PERCENTAGE SURVIVAL RESPONSES AT VARIOUS END POINTS IN ADRENALECTOMIZED RATS TREATED AT VARIOUS DOSAGES OF COMPOUND "F" AND IN UNTREATED SHAM-ADRENALECTOMIZED CONTROLS

End points	Mean responses at dosages (micrograms) of compound "F"						Untreated sham-operated
	0	15	25	50	100	200	
15 minutes	20.3 (4.7)	41.0 (4.9)	67.0 (4.8)	81.5 (13.5)	82.9 (5.1)	90.2 (3.3)	100 (0)
20 minutes	12.8 (3.4)	28.7 (4.3)	56.5 (5.5)	75.3 (12.0)	75.3 (7.4)	89.4 (3.5)	100 (0)
30 minutes	8.1 (2.4)	28.7 (4.3)	43.4 (5.5)	56.5 (9.0)	70.4 (8.8)	87.7 (3.8)	100 (0)
60 minutes	4.8 (2.0)	18.0 (1.0)	36.5 (5.1)	52.3 (10.8)	65.7 (9.7)	83.5 (4.5)	100 (0)
120 minutes	4.8 (2.0)	18.0 (1.0)	33.7 (4.9)	48.3 (7.2)	60.9 (10.7)	81.6 (4.7)	100 (0)
18 hours	0.8 (0.5)	5.7 (5.7)	20.9 (3.7)	26.0 (3.4)	32.3 (9.4)	65.6 (5.2)	100 (0)
Total Number of Animals	103	17	136	23	39	116	12

Values shown in parentheses represent standard deviations of the means.

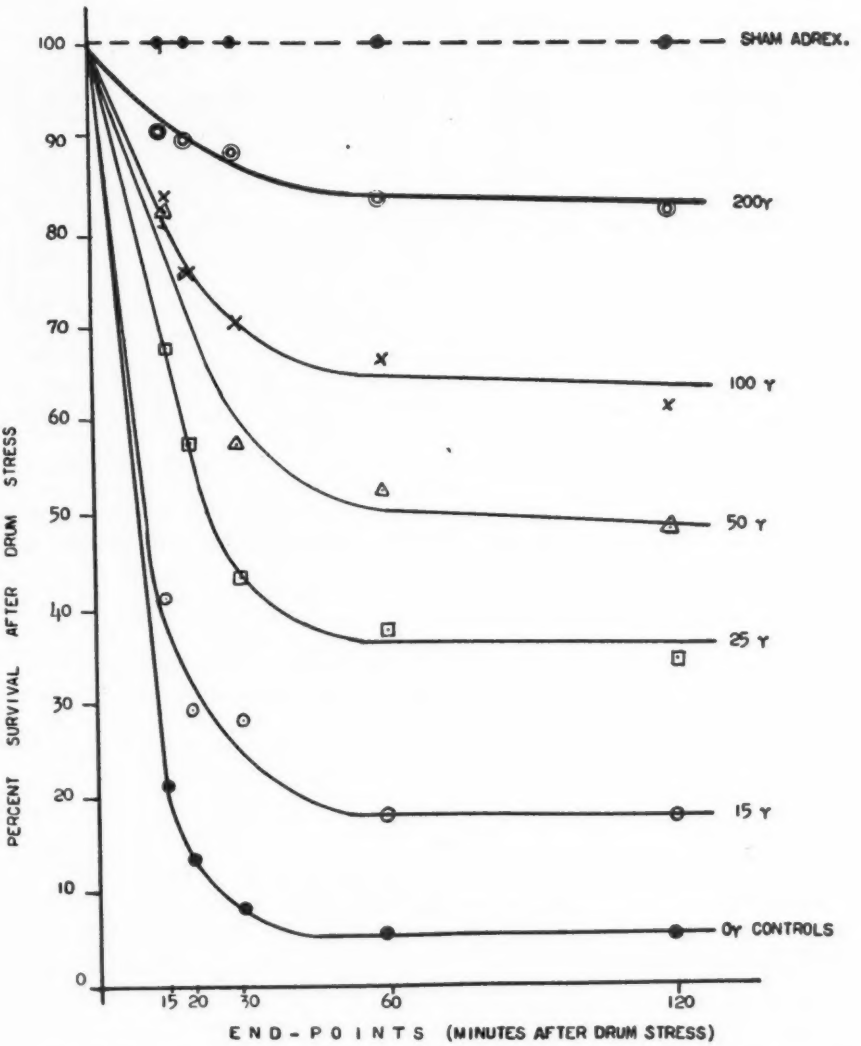

FIG. 2. Mean percentage survival responses at various end points in adrenalectomized rats treated at various dosages of compound F and in untreated sham-adrenalectomized controls.

TABLE 2. COMPOUND "F": VALUES AND STANDARD DEVIATIONS FOR SLOPE AND INTERCEPT AT SEPARATE END POINTS

End point	Slope (b)	S.D.$_b$	Intercept (a)	S.D.$_a$
15 minutes	30.938	2.597	20.626	4.233
20 minutes	33.239	2.676	10.753	4.362
30 minutes	33.730	2.704	3.091	4.406
60 minutes	33.254	2.868	−1.349	4.675
120 minutes	32.060	2.865	−1.722	4.669
18 hours	25.501	2.738	−6.224	4.463
Mean Values	31.453	2.741	4.196	4.468

The calculated values for the slope (b) and for the intercept (a), together with their respective standard deviations are shown in Table 2. From these data it will be seen that the values of the slope obtained from the different end point analyses seem to differ from each other no more than would be expected from random fluctuation. As would be expected, the intercept (a) takes on progressively smaller values as the end point intervals increase.

In attempting further to discover a means whereby to utilize the available data as effectively as possible, subsequent statistical studies revealed that greater accuracy could be obtained by combining the observations for all end points in the calculation of a single, over-all regression. Using this technique, the total variation is broken down and ascribed to various specific sources which, in this case are shown to consist of:

A. Differences in response due to observations at different end points.
B. Differences in response due to different dosage treatments.
C. Interaction between end point and dosage-treatment responses.
D. Variability within groups.

Sources C and D combined afford the best estimate of experimental error and are used to test the significance of the other sources of variability.

(The partitioning of total variation in this manner is not unique, although it appears that it has not been applied previously in the case of percentage-survival responses). The total variation may be further partitioned as follows:

A. Variation due to regression.
B. Total variation about the regression line.

It was found that a significant portion of the total variation could be ascribed to the linear relationship between response and log dosage. The total variance about the regression line is used in the calculation of an estimate of the standard deviation of the slope and of the intercept. Following this procedure of analysis the equation for the regression line for treatment with compound "F" was found to be: $R=(4.199\pm2.055)+(31.452\pm1.608)x$ (see Table 3).

The improvement afforded by the use of the composited values of all observations over that based upon separate end point determinations is clear from the decrease of more than 40% in both constants in the equation seen by comparison of the standard deviations (Table 2) for slope and intercept at the various end points with those shown in above regression equation. Both linearity and sensitivity in the dosage-response with respect to compound "F" are established by the high degree of significance (0.001 level) in both these factors as shown in Table 3.

Bio-assay of other corticosteroids.—Having established the linearity in

TABLE 3. COMPOUND "F"—ANALYSIS OF VARIANCE: COMPOSITED DATA FOR ALL END POINTS

Sources of variation	Deviance	Degrees freedom	Unbiased est. of variance	F	Degree of Significance
Differences among	(Q)	(ν)	(Q')		
A. Mean End Point Responses	52,818.45	5	10,563.69	28.2	****
B. Mean Responses to Treatments	341,944.52	5	68,388.90	182.6	****
C. Interaction A.B	11,247.00	25	449.88	1.2	N.S.
D. Variation within Groups	150,937.43	408	369.945	—	—
Totals	556,947.40	443	—	—	—
Error (C+D)	162,184.43	433	374.560	—	—
Regression of Response on log (Dose+1)	324,069.70	1	324,069.70	865.2	****
Total Variation about Regression Line	232,877.70	432	539.609		

Equation for the Regression Line: $R = (4.199 \pm 2.055) + (31.452 \pm 1.608)x$

**** = significant at the 0.001 level.
N.S. = not significant.

dosage-response in the case of compound "F," further experiments were conducted to test these findings as to their application in the bio-assay of compounds known to possess adrenocortical activity. The substances assayed were—adrenocortical extract; cortisone (cpd. E); and corticosterone (cpd. B).

The adrenalectomized animals used in any single test were segregated into four groups with as even a distribution as possible. Two of these groups received compound "F," one at a low dosage (S_1), the other, (S_2) at a higher dosage. The remaining groups were similarly divided according to low and high dosages of the substance being assayed and designated respectively as U_1 and U_2. The respective dosage ratios for standard and unknown were computed so as to be identical, as demand by the statistical procedure adopted: i.e., $S_2:S_1 = U_2:U_1$.

Bioassay of adrenocortical extract.—Four separate tests were conducted using a sample of hog adrenal extract solids[1] containing activity equivalent to that of 100 mg. of compound "F" as measured by the liver glycogen deposition bio-assay method. The test solutions were prepared in 20% ethanol on the day of injection at dosages of 25 and 200 micrograms of compound "F"-equivalent per rat. Identical amounts of compound "F" standard were run simultaneously in the control groups.

Table 4 shows the mean values of the responses observed at the various dosages of treatment and at the six end points used. These data were sub-

[1] Supplied through the courtesy of the Upjohn Company, Research Laboratories, Kalamazoo, Michigan.

TABLE 4. BIO-ASSAY OF ADRENOCORTICAL EXTRACT (HOG)*

Mean Percentage Survival Responses at Various End Points in Cpd. "F" Controls and A.C.E.-Treated Rats

End points	Percentage survival at dosages (micrograms) of			
	"F" @ 25	"F" @ 200	A.C.E. @ 25	A.C.E. @ 200
15 minutes	63	98	62	93
20 minutes	52	98	52	93
30 minutes	52	91	38	89
60 minutes	52	91	38	89
120 minutes	43	91	38	89
18 hours	38	83	29	86
Total Number of Animals	27	25	28	27

* The dosage levels as expressed in terms of Cpd. "F" for this extract were computed from the suppliers' stated "F"-equivalent activity based upon bio-assay using the Liver Glycogen Deposition method. The material used as designated as "Lot No. 906—Hog Adrenal Extract Solids—Upjohn Co., Research Labs."

jected to variance analysis using the same general technique as that employed in the case of compound "F."

The calculated values for slope, intercept and relative potency together with their respective standard deviations at each end point are shown in Table 5. From these data it will be seen that the relative potency values show a wide variation (112.8%–143.7%) with very large standard deviations (54.4%–72.8%) from which it is clear that the confidence limits are much too wide for an accurate estimate of potency in terms of compound "F."

When however, all data are combined as in the case of the previous analysis for compound "F," and the sources of variation isolated to correct for: A. Differences among responses to dosage treatments; B. Differences among endpoint responses; C. Differences among responses in the individual

TABLE 5. BIO-ASSAY OF ADRENOCORTICAL EXTRACT

Values and Their Standard Deviations at Separate End Points for Slope, Intercept, and Relative Potency

End point	Slope (b)	S.D.$_b$	Intercept (a)	S.D.$_a$	Relative potency	S.D.$_{rp}$	Index of precision (λ)
15 minutes	36.403	9.549	11.361	18.252	118.1	60.6	0.44
20 minutes	47.891	10.930	−15.074	20.891	112.8	56.0	0.43
30 minutes	49.967	11.176	−24.975	21.362	143.7	72.8	0.43
60 minutes	49.967	11.176	−24.975	21.362	143.7	72.8	0.43
120 minutes	54.812	11.189	−36.124	21.387	115.8	54.4	0.41
18 hours	56.058	11.399	−44.865	21.780	113.7	55.8	0.43
Mean Values	49.183	10.903	−22.442	20.841	—	—	—

TABLE 6. BIO-ASSAY OF ADRENOCORTICAL EXTRACT—ANALYSIS OF VARIANCE: COMPOSITED DATA FOR ALL END POINTS

Sources of variation	Deviance (Q)	Degrees freedom (ν)	Unbiased est. of variance (Q')	F	Degree of significance
Differences among					
A. Mean End Point Responses	3,767.3	5	753.46	12.20	***
B. Mean Responses to Treatments	48,039.4	3	16,013.10	259.20	***
C. Mean Responses in Individual Tests	6,608.8	3	2,202.90	35.66	***
D. Interaction A.B	1,287.3	15	85.82	1.46	N.S.
E. Interaction A.C	701.4	15	46.76	0.80	N.S.
F. Interaction B.C	21,549.5	9	2,394.40	40.75	***
G. Interaction A.B.C	2,644.3	45	58.76		
Totals	84,598.0	95	—	—	—
Error (G+E+D)	4,633.0	75	61.773	—	—
Regression of Response on log (Dosage)	47,348.2	1	47,348.2	119.15	***
Response Difference ("F" vs. A.C.E.)	504.2	1	504.2	8.16	**
Slope Difference ("F" vs. A.C.E.)	187.0	1	187.0	3.03	N.S.

Equation for the Regression Line: $R = (49.183 \pm 4.499)x - (22.418 \pm 8.569)$
Potency (Relative to Compound "F") = 123.9% ±9.4%
Confidence Limits for Relative Potency: (0.05 level) = $106.6 < P < 144.0$
Index of Precision of the Assay (λ) = 0.16

*** = significant at the 0.005 level.
** = significant at the 0.01 level.
N.S. = not significant.

tests; and the interaction factors included as before, it is seen that a significant (0.01 level) difference between standard and unknown is demonstrable with no significant differences in respective slopes. The data are summarized in Table 6 showing the regression equation as: $R = (49.183 \pm 4.499)x - (22.418 \pm 8.569)$, the relative potency of A.C.E. in terms of cpd. "F" as 123.9% ±9.4% with confidence limits at the 0.05 level given by: $106.0 < P < 144.0$, and the index of precision (λ) as 0.16. Thus, on the basis of the liver glycogen units employed, this sample of A.C.E. was found to be 80.7% as effective as compound "F" in its protective action against drum stress.[2]

It is of interest to note the degree of improvement seen by making use of the combined data in preference to the separate end point responses in the statistical analysis of the assay. As in the case of compound "F," the variation in both equation coefficients is greatly reduced; thus, the stand-

[2] In order to elicit a response equivalent to that produced by 100 units of compound "F," 123.9 units of A.C.E. are required, i.e., A C.E. is 100/123.9 or 80.7% as potent as compound "F."

TABLE 7. BIO-ASSAY OF "CORTISONE" (COMPOUND "E"—KENDALL)
Mean Percentage Survival Responses at Various End-Points in Compound "F" Controls and in Cortisone-Treated Rats

End points	Percentage survival at dosages (micrograms/rat) of			
	"F" @ 25	"F" @ 200	"E" @ 25	"E" @ 200
15 minutes	89	100	60	86
20 minutes	74	100	55	81
30 minutes	69	100	45	81
60 minutes	48	100	45	76
120 minutes	48	100	45	71
18 hours	37	71	26	66
Total Number of Animals	17	18	17	21

ard deviation for slope decreases from 10.903 to less than half that value, 4.499, while a similar reduction of 20.481 to 8.569 is seen in the case of the intercept. The index of precision for the assay is likewise improved from 0.43 to 0.16. In view of these findings, this technique of variance analysis was adopted and in the subsequent assays the separate end point data were no longer analysed *per se*.

Bio-assay of cortisone (*cpd. "E"*): Three separate tests were conducted using crystalline cortisone in 20% aqueous ethanol, prepared and administered according to the standardized procedure. The dosages used in all three tests were 25 and 200 micrograms per rat, both in the case of the standard and the unknown.

The end-point responses and analysis of variance are shown in Tables 7 and 8. From these data it is seen that a highly significant difference between standard and unknown is demonstrable with no significant difference in the respective slopes. The equation for the regression line is given by: $R = (2.708 \pm 9.936) + (36.234 \pm 5.217)x$. The relative potency becomes $284.3\% \pm 42.4\%$ with limits of confidence at the 0.05 level as $210.9 < P < 383.1$. The index of precision for the assay is 0.245. The protective action of cortisone against fatal drum stress is therefore 35.17% that of compound "F."

Bio-assay of corticosterone (*cpd. "B"*): Crystalline corticosterone was used in the three tests comprising this assay at dosages of 50 and 200 micrograms per rat. The standard was run at these same dosage levels.

The mean values for all responses are shown in Table 9, and the data for the analysis of variance in Table 10. A highly significant difference between standard and unknown is shown with no significant difference in the respective slopes. The equation for the regression line is: $R = (53.71 \pm 9.38)x - (47.59 \pm 19.0)$. The relative potency with respect to compound "F" is shown to be $239.7\% \pm 32.62\%$ with confidence limits (0.05 level) given by:

TABLE 8. BIO-ASSAY OF CORTISONE—ANALYSIS OF VARIANCE: COMPOSITED DATA FOR ALL END POINTS

Sources of variations	Deviance (Q)	Degrees freedom (ν)	Unbiased est. of variance (Q')	F	Degree of significance
Differences among					
A. Mean End Point Responses	8,031.44	5	1,606.288	20.33	****
B. Mean Responses to Treatments	24,194.33	3	8,064.777	102.10	****
C. Mean Responses in Individual Tests	450.11	2	225.055	2.85	N.S.
D. Interaction A·B	2,603.34	15	173.556	2.30	*
E. Interaction A·C	894.89	10	89.489	1.18	N.S.
F. Interaction B·C	8,800.33	6	1,466.722	19.42	****
G. Interaction A·B·C	2,266.00	30	75.533	—	—
Totals	47,240.44	71	—	—	—
Error: (G+E)	3,160.89	40	79.022	—	—
Regression of Response on log (Dosage)	19,273.39	1	19,273.390	243.9	****
Response Difference ("F" vs. "E")	4,867.56	1	4,867.56	61.6	****
Slope Difference ("F" vs. "E")	53.389	1	53.389	0.68	N.S.

Equation for the Regression Line: $R = (2.708 \pm 9.936) + (36.234 \pm 5.217)x$
Potency (Relative to Compound "F") = 284.3% ± 42.4%
Confidence Limits for Relative Potency (0.05 level) = $210.9 < P < 383.1$
Index of Precision of the Assay (λ) = 0.245

**** = significant at the 0.001 level.
* = significant at the 0.05 level.
N.S. = not significant.

$182.6 < P < 314.7$. The index of precision $\lambda = 0.212$. Corticosterone is thus shown to have 41.7% the activity of compound "F."

SUMMARY

A method of bioassay of the "stress-survival" type for the evaluation of

TABLE 9. BIO-ASSAY OF CORTICOSTERONE (COMPOUND "B"—KENDALL)

Mean Percentage Survival Responses at Various End Points in Compound "F" Controls and in Corticosterone-Treated Rats

End points	Percentage survival at dosages (micrograms/rat) of			
	"F" @ 50	"F" @ 200	"B" @ 50	"B" @ 200
15 minutes	71	96	57	78
20 minutes	67	93	48	78
30 minutes	63	88	42	68
60 minutes	49	88	34	58
120 minutes	45	86	25	58
18 hours	19	77	4	44
Total Number of Animals	21	22	21	22

TABLE 10. BIO-ASSAY OF CORTICOSTERONE—ANALYSIS OF VARIANCE: COMPOSITED DATA FOR ALL END POINTS

Sources of variations	Deviance (Q)	Degrees freedom (ν)	Unbiased est. of variance (Q')	F	Degree of significance
Differences among					
A. Mean End Point Responses	12,266.167	5	2,453.233	18.902	****
B. Mean Responses to Treatments	26,507.444	3	8,834.814	68.073	****
C. Mean Responses to Individual Tests	10,492.750	2	5,246.375	40.424	****
D. Interaction A.B	1,787.722	15	119.181	0.974	N.S.
E. Interaction A.C	1,679.583	10	167.958	1.372	N.S.
F. Interaction B.C	2,549.472	6	424.912	3.473	***
G. Interaction A.B.C	3,670.862	30	122.362	—	—
Totals	58,954.000	71	—	—	—
Error: (G+E+D)	7,138.167	55	129.785	—	—
Regression of Response on log (Dosage)	18,818.208	1	18,818.208	145.034	****
Response Difference ("F" vs. "B")	7,482.722	1	7,482.722	57.655	****
Slope Difference ("F" vs. "B")	206.722	1	206.722	1.593	N.S.

Equation for the Regression Line: $R = (53.71 \pm 9.38)x - (47.59 \pm 19.00)$
Potency (relative to cpd. "F") = 239.70% ± 32.62%
Confidence Limits (for Rel. Potency: 0.05 level) = $182.6 < P < 314.7$
Index of Precision for the Assay (λ) = 0.212

**** = significant at the 0.001 level.
*** = significant at the 0.005 level.
N.S. = not significant.

adrenocortical activity in biological materials and in crystalline compounds is described.

The standardized test stimulus comprises trauma with shock experimentally induced by means of a modified form of the "Noble-Collip Drum." Such trauma can be reproduced from test to test as to kind, degree and duration.

The "response," from which is derived the relative potency, is expressed as the percentage survival following cessation of drum stress in fasting, pre-treated adrenalectomized male albino rats. The potency of the unknown test material is computed in terms of its response relative to that elicited by the standard of reference, compound "F," which is significantly active at a dosage level of 15 micrograms.

Application of the method in the bio-assay of three substances known to possess adrenocortical activity shows that adrenocortical extract (A.C.E.) offers 80.7%, cortisone (cpd. "E") 35.17%, and corticosterone (cpd. "B") 41.7% of the protection against fatal traumatic shock as does hydrocortisone (cpd. "F").

Further studies are in progress to determine the relative potency of other steroid substances by this method of assay.

Acknowledgements

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PLASMA PROTEIN-BOUND IODINE AFTER INTRAVENOUS INJECTION OF THYROXINE IN THYROIDECTOMIZED DOGS[1]

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THERE is abundant evidence that circulating thyroid hormone is chiefly thyroxine which is bound to plasma protein. Almost all of the endogenously produced organic iodine bound to plasma protein is present as thyroxine (Taurog and Chaikoff, 1948; Laidlaw, 1949; Gross *et al.*, 1950; Rosenberg, 1951; Taurog *et al.*, 1950; Tong *et al.*, 1952). When thyroxine is added to serum it becomes protein-bound. The resulting thyroxine-protein complex behaves on electrophoresis like the thyroid hormone—plasma protein complex (Gordon *et al.*, 1952) indicating an identical protein linkage. O'Neal and Heinbecker (1953) have presented rate-constants for the disappearance of endogenously produced protein-bound iodine from plasma after thyrotropin stimulation in hypophysectomized dogs. It is the purpose of the present report to present the characteristics of the disappearance of protein-bound iodine from plasma after intravenous injection of racemic thyroxine in thyroidectomized dogs.

MATERIALS AND METHODS

Healthy young adult mongrel dogs were used. The diet was Purina dog chow containing 0.5% iodized salt, and supplemental ration of fresh horse meat. Thryoidectomies were done surgically. In one instance, a hypophysectomized dog was used. The hypophysis of this dog was removed via the oral approach.

Plasma protein-bound iodine determinations were done by a modification (O'Neal and Simms, 1953) of the chloric acid method of Zak *et al.* (1952). Protein-bound iodine concentrations are expressed in micrograms per 100 ml. of plasma (μg.%).

Following thyroidectomy or hypophysectomy, after the plasma protein-bound iodine concentrations had fallen to levels near 1 μg.%, single intravenous injections of 500 μg. or of 5000 μg. of d,l-thyroxine (Squibb) were given. Venous blood was drawn for plasma protein-bound iodine determinations at frequent intervals until the concentrations fell to low levels. No samples were taken from the vein used for thyroxine injection.

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RESULTS

1. *Plasma protein-bound iodine concentrations after intravenous injection of 500 μg. of d,l-thyroxine.*

The two thyroidectomized dogs were each given an intravenous injection of 500 μg. of d,l-thyroxine on the 17th day after thyroidectomy. Serial determinations of plasma protein-bound iodine concentration were then done. Later, one of the dogs (Thy 1) was given 100 mg. of cortisone acetate (Merck) intramuscularly daily. On the third day of cortisone therapy, an intravenous injection of 500 μg. of d,l-thyroxine was given and serial determinations of plasma protein-bound iodine again done. Daily injections of 100 mg. of cortisone acetate were continued until plasma protein-bound iodine concentrations fell to low levels. The results of these experiments are presented in Figure 1. After injection of 500 μg. of thyroxine, protein-bound iodine at first disappeared from plasma rapidly and then a slower rate of disappearance became apparent. Finally, on the fourth or fifth day, low concentrations which did not differ significantly from the pre-injection concentrations were reached.

The initial rapid disappearance of protein-bound iodine from plasma is believed to be due to the distribution of thyroxine from plasma into tissues and body fluids in addition to actual disappearance due to metabolic destruction and excretion. The later slower phase is attributed to metabolic destruction and excretion after distribution is complete. It is possible to derive the kinetic characteristics of each of these processes and the distribution space of thyroxine, by means of concepts previously developed and applied to other substances (Dominguez *et al.*, 1947; Burch *et al.*, 1947; Albert and Keating, 1949).[2] The method of analysis is illustrated in

[2] The curves of disappearance of organic iodine from plasma follow the general equation $PBI - PBI\ equil = a_1e^{-b_1t} + a_2e^{-b_2t}$, where (Figure 2):

PBI = measured protein-bound iodine concentration

PBI equil = final protein-bound iodine concentration

a_1 = concentration (μg%) of the more rapid regression (D) at zero time, by extrapolation

b_1 = slope (rate constant) of curve D.

$$= \left(\frac{\log_{10} y_2 - \log_{10} y_1}{t_2 - t_1} \right) 2.3$$

a_2 = concentration (μg%) of the slower regression (B) at zero time, by extrapolation

b_2 = slope of curve B

t = time in hours

e = the base of the natural logarithm

Calculation of the volume of distribution of thyroxine is based on a concentration of a_2 under conditions of instantaneous distribution: (*Continued on page 361*)

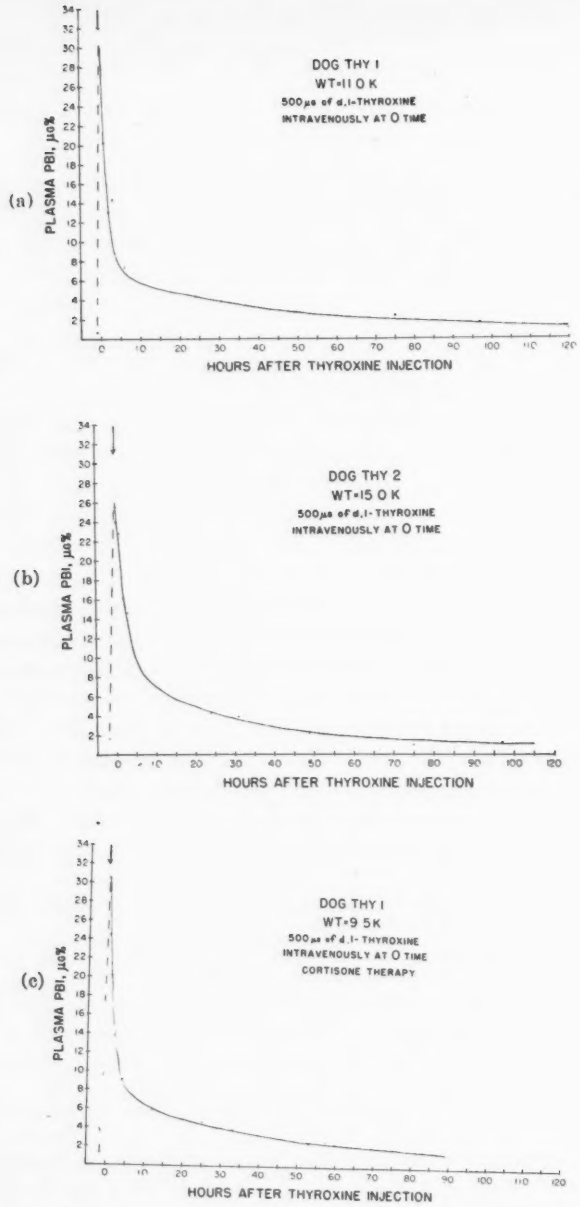


FIG. 1a, b. Plasma protein-bound iodine concentrations after intravenous injection of 500 μg. of d,l-thyroxine in two thyroidectomized dogs.

FIG. 1c. Plasma protein-bound iodine concentrations after intravenous injection of 500 μg. of thyroxine in a thyroidectomized dog given cortisone. Cortisone injections (100 mg. daily intramuscularly) were begun 3 days before and continued until the fifth day after thyroxine injection.

Figure 2. The characteristics of the "distribution" curve D and of the "disposal" curve B are presented in Table 1.

TABLE 1. KINETIC CHARACTERISTICS OF THE DISAPPEARANCE OF ORGANIC IODINE FROM PLASMA AFTER THYROXINE INJECTION (500 μg.)

Dog	Wt., Kg.	Treatment	Pre-injection PBI, μg.%	PBI equil, μg.%	Distribution curve D			Disposal curve B			V.D.	
					a_1	b_1	$B_{1/2}{}^1$	a_2	b_2	$B_{1/2}{}^2$	ml.	% B.W.
Thy 1	11.0	500 μg. thyroxine	0.7	1.0	25.8	0.715	0.97	6.2	0.025	27.7	5260	47.8
Thy 2	15.0	500 μg. thyroxine	1.7	1.0	17.0	0.317	2.15	8.5	0.038	18.3	3980	26.5
Thy 1	9.5	500 μg. thyroxine +cortisone	1.3	1.2	24.0	0.600	1.15	6.6	0.025	27.7	4990	52.5

The definitions of abbreviations are given in Footnote 2.

It is obvious that in the single experiment with concurrent administration of cortisone and thyroxine, cortisone did not influence the rate of distribution, the volume of distribution, or the rate of disposal of injected racemic thyroxine.

2. *Plasma protein-bound iodine concentrations after intravenous injection of 5000 μg. of d,l-thyroxine.*

Two thyroidectomized dogs (Thy 1 and Thy 2, the same dogs used in the experiments just described) and one hypophysectomized dog (K97) were each given an intravenous injection of 5000 μg. of d,l-thyroxine. The injection was given to K97 ten days post-hypophysectomy. Serial determinations of plasma protein-bound iodine were then done. The results are shown in Figure 3, where the actual values are plotted on semilogarithmic coordinates.

Whereas the disappearance of protein-bound iodine from plasma after the injection of 500 μg. of thyroxine follows two simple regressions, the curve of disappearance after the injection of 5000 μg. of thyroxine appears to be the result of multiple regressions. A major difference between the curves obtained with the smaller dose (500 μg.) and with the larger dose (5000 μg.) is that with the latter dosage the rate of disappearance of plasma protein-bound iodine after concentrations reach values near 3–4 μg.% appears to be extremely slow. If a curve of this final extremely slow disappearance of precipitable iodine from plasma is constructed and the values

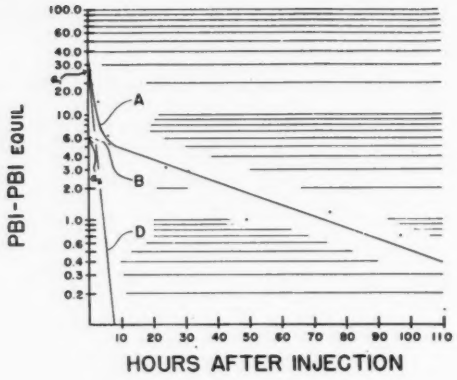
FOOTNOTE 2 (*Continued*)

$$\text{V.D.} = \frac{\text{Dose}}{a_2/0.653} \div 100$$

where V.D. = volume of distribution, ml.
Dose = injected thyroxine, μg.
0.653 = fraction of thyroxine which is iodine, by wt.

The biological half-time value ($B_{\frac{1}{2}}$) is calculated by dividing 0.693, the natural logarithm of 2, by the rate constant.

(a)



(b)

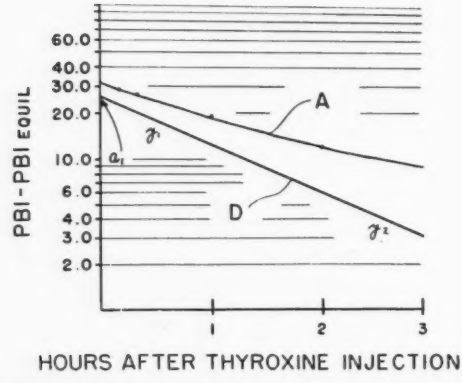
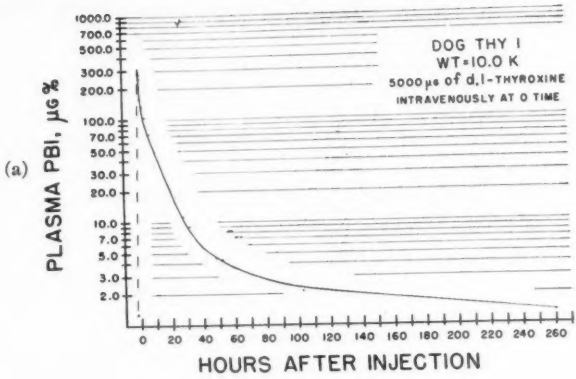


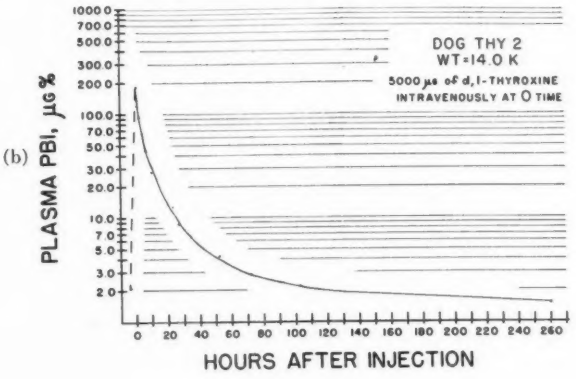
FIG. 2a. Method of analysis of curve of disappearance of organic iodine from plasma after intravenous injection of 500 μg. of thyroxine. The A curve is plotted on the semi-logarithmic coordinate from the data shown in Figure 1a, minus the final equilibrium value. The final slower regression (curve B) is assumed to represent disappearance of organic iodine from plasma via metabolic and excretory routes, and curve D (calculated by difference: $D_t = A_t - B_t$) represents distribution of thyroxine. The points a_1 and a_2 are protein-bound iodine concentrations at zero time, obtained by extrapolation of the two regressions D and B, respectively.

FIG. 2b. The early portion of the curve shown in Figure 2a. The points y_1 and y_2 are any points on the D curve at t_1 and t_2 times.

along this curve are subtracted from (PBI − PBI equil), then the remainder of the curve is susceptible to analysis in the same manner as described for the curves in the previous section. While determinations in the very low range were too few to accurately characterize the kinetics of the final slow regression, except in the case of the hypophysectomized dog, this regression appeared in all three experiments.

The characteristics of the regressions ascribed to distribution and to





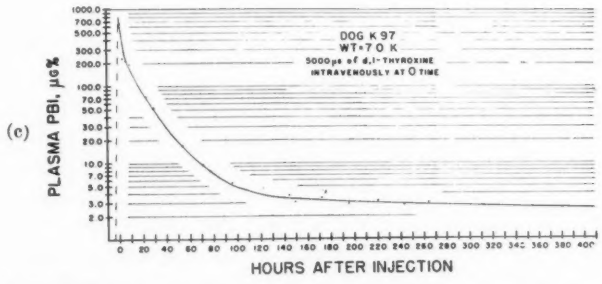


FIG. 3a, b. Plasma protein-bound iodine concentrations after intravenous injection of 5000 µg. of thyroxine in two thyroidectomized dogs. These are semilogarithmic plots with time on the arithmetic abscissa and protein-bound iodine concentration on the logarithmic ordinate.

FIG. 3c. Plasma protein-bound iodine concentrations after intravenous injection of 5000 µg. of thyroxine in a hypophysectomized dog.

TABLE 2. KINETIC CHARACTERISTICS OF THE DISAPPEARANCE OF ORGANIC IODINE FROM PLASMA AFTER THYROXINE INJECTION (5000 μG.)

Dog	Wt., Kg.	Pre-injection PBI, μg.%	PBI equil. μg.%	Distribution curve D			Disposal curve B			Unknown curve X			V.D.	
				a_1	b_1	$B_{1/2}{}^1$	a_2	b_2	$B_{1/2}{}^2$	a^3	b^3	$B_{1/2}{}^3$	ml.	% B.W.
Thy 1	10.0	1.3	1.3	181	1.173	0.59	125	0.103	6.7	2.9	0.008	85.5	4100	41.0
Thy 2	14.0	2.1	1.5	98.5	0.557	1.24	68.5	0.089	7.8	1.7	0.007	100	4780	34.1
K97	7.0	0.7	1.7	590	0.419	1.65	215	0.051	13.6	2.1	0.002	315	1520	21.7

The definitions of abbreviations are given in Footnote 2.

disposal, and of the very slow final regression (curve X) following the intravenous injection of 5000 μg. of thyroxine are presented in Table 2.

DISCUSSION

The rate of distribution of thyroxine from plasma into its volume of distribution had an average half-time of 1.29 hours ± S.D. 0.55 hours in six determinations. There were no significant differences between the rate of distribution after injection of 500 μg. of thyroxine and the rate of distribution of 5000 μg. Benua *et al.* (1952) found that distribution of radiothyroxine in hyperthyroid patients had a half-value time of 3.3 hours.

The disappearance of protein-bound iodine from plasma after distribution is complete (curve B) is due to metabolic destruction of thyroxine and to excretion of thyroxine in feces and urine. The relative part played by each of these processes in our experiment is not known. Curve B is considered to represent only "disposal" of thyroxine by all routes. This second slower phase of disappearance of precipitable iodine from plasma had half-life times of 6.7, 7.8 and 13.6 hours after the intravenous injection of 5000 μg. of d,l-thyroxine in 3 experiments, and half-times of 27.7, 18.3 and 27.7 hours after the injection of 500 μg. These times may be compared with a mean half-life for the disappearance of endogenously produced thyroid hormone after thyrotropin stimulation in hypophysectomized dogs of 16.2 hours (O'Neal and Heinbecker, 1953). The dog disposes of injected thyroxine at a much more rapid rate than does the human. Albert and Keating (1949) found the biological half-life of precipitable radioactivity in plasma after the injection of radioactive thyroxine in a cretin to be 3.8 days. In hyperthyroid patients (Benua *et al.*, 1952) half-time values for disposal of radiothyroxine averaged 5.7 days. Recant and Riggs (1952) reported biological half-times of plasma protein-bound iodine after intravenous injection of thyroxine to be 4.3 days in a normal subject, 4.3 days in a nephrotic subject, and 8.3 days in a patient with untreated myxedema.

Because of an increased rate of disposal after administration of the larger dosage of thyroxine, the dog is able to rid itself of the larger dose in about the same time it disposes of the smaller dose. Calculation from the plasma protein-bound iodine concentrations and the volume of distribution shows that the dogs receiving 5000 μg. of thyroxine had an average of 300 μg. of thyroxine remaining in blood and tissues at 50 hours, while the dogs that

received 500 μg. had an average of 113 μg. remaining at 50 hours. Thus at 50 hours, the dogs had disposed of 94.1% of the larger dose and 75.4% of the smaller dose. The more rapid rate of disposal of the larger amount of thyroxine may indicate that thyroxine accelerates its own metabolic destruction, or that excretion proceeds at a more rapid rate with the larger dosage.

The significance of the final extremely slow regression (curve X) after injection of 5000 μg. of thyroxine is not clear. Gross and Leblond (1947) found that when large doses of thyroxine were injected into rats, a large proportion of the hormone appeared in feces as organic iodine. Others have shown (Albert and Keating, 1949; Benua *et al.*, 1952) in the human that when more physiological amounts of thyroxine are injected, only a relatively small fraction of the administered dose appears in feces. It is possible that the slow regression is due to biliary excretion of much of the injected hormone, followed by an enterohepatic cycle and gradual return of a small amount of the hormone to the systemic circulation. Further study of the fate of injected thyroxine is needed to elucidate this point.

The mean volume of distribution of 3,l-thyroxine was found to be 36.9 ±S.D. 11.8% of body weight. Thus the volume of distribution in the dog agrees fairly well with that in the human. Benua *et al.* (1952) found the volume of distribution to average 34.1% of body weight in hyperthyroid subjects. Myant and Pochin (1950) found the thyroxine space to be 32.6% of body weight in normal human subjects.

An increased rate of metabolic destruction of thyroxine must be considered as a possible explanation for the decreases in plasma protein-bound iodine concentrations which have been observed during cortisone therapy in the human (Hardy *et al.*, 1950; Frederickson *et al.*, 1952) and in the rat (Foster, 1952). Bondy and Hagewood (1952) have found, on the contrary, that cortisone may reduce the rate at which thyroxine is removed from plasma in rats. The single experiment in which cortisone did not influence the rate of disappearance of thyroxine from plasma in a thyroidectomized dog does not justify specific conclusions. However, this observation, considered together with the previous observation (O'Neal and Heinbecker) that cortisone did not influence the rate of disappearance of endogenously produced thyroid hormone from plasma, may indicate that cortisone does not increase metabolic destruction of thyroxine in the dog.

SUMMARY

A single dose of racemic thyroxine was injected intravenously into dogs. A dosage of 500 μg. of thyroxine was given to thyroidectomized dogs, and a dosage of 5000 μg. was given to one hypophysectomized and two thyroidectomized dogs. After the intravenous injection of 500 μg. of thyroxine the disappearance of protein-bound iodine from plasma had two phases. The initial rapid phase of disappearance, with an average half-time of 1.42

hours, is attributed to the distribution of thyroxine into tissues and body fluids. The slower phase of disappearance of protein-bound iodine from plasma following distribution had an average half-time of 24.6 hours. This phase is believed due to metabolic destruction and excretion of thyroxine.

When a dosage of 5000 μg. of thyroxine was administered, the character of the curve of disappearance of protein-bound iodine from plasma indicates multiple regressions. Analysis of these curves indicates that the rate of distribution (mean half-time = 1.16 hours) was about the same as with the smaller dose of 500 μg. The rate of disposal, with a half-value time averaging 9.4 hours, was very much more rapid than the rate of disposal after injection of 500 μg. of thyroxine.

Six determinations of thyroxine space in three dogs averaged 36.9 ± 11.8 per cent of body weight.

The administration of cortisone did not influence the rate of distribution, the volume of distribution or the rate of disposal of injected racemic thyroxine in a thyroidectomized dog.

Acknowledgements

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DEMONSTRATION OF STORAGE AND RELEASE OF GONADOTROPIN BY THE ANTERIOR PITUITARY OF THE MOUSE DURING GESTATION[1,2]

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IN A previous study in which pituitaries were assayed for gonadotropic activity by induction of ovulation in pregnant female mice (Ladman and Runner, 1951a), differences in the responsiveness of assay animals seemed to be dependent on the stage of pregnancy. To test this conclusion more fully, an investigation of the ovulatory response of the pregnant mouse to pituitary gonadotropin was undertaken, utilizing a greater number of gestational stages (Ladman and Runner, 1951b). In addition to the bioassays, the pituitaries of the graded series of animals were studied histologically.

In the course of this study it became evident that our experimental animals which served not only as the source of pituitary gonadotropin but also for the assay of gonadotropin, presented an unusual opportunity to evaluate two current concepts of pituitary function in a species which had not been investigated heretofore. These hypotheses of pituitary function are: (1) that the anterior pituitary is relatively inactive with respect to manufacture and release of gonadotropins during pregnancy, and (2) that variation in gonadotropic content may denote either a change in rate of production or an alteration in rate of discharge (cf. Severinghaus, 1939;

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[2] This paper is a revision of a thesis presented to the Graduate School, Indiana University in partial fulfillment of the requirements for the degree Ph.D. in Anatomy (AJL).

[3] Fellow of the American Cancer Society, Inc. recommended by the Committee on Growth of the National Research Council, 1952–1953.

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Smith, 1939). In regard to the first question, the results of the present experiments indicate that the anterior pituitary of the mouse during pregnancy is a very active gland. In regard to the second question, the results indicate that measurement of the degree of pituitary activity depends upon quantitative variation in storage and release of pituitary gonadotropin (LH).

MATERIALS AND METHODS

Female mice of the Swiss strain, 3 to 6 months of age, were utilized. They were fed a diet of Purina Fox Checkers, supplemented weekly with rolled oats. Water was available *ad libitum*. Males were introduced into the cages at the onset of the experiment, and the females were checked each morning for the presence of copulatory plugs. Animals showing evidence of having mated were isolated and classified as ½ day pregnant, since ovulation in cyclic female mice normally occurs between 12 midnight and 2 A.M. (Snell, Fekete, Hummel and Law, 1940). This manner of establishing the beginning of gestation was employed throughout this investigation. All animals in this study were in their first pregnancy.

Pituitary glands were removed from donor animals at 6½, 9½, 12½, 15½, and 18½ days of pregnancy and were weighed on a Roller-Smith Torsion Balance, sensitive to 0.1 mg. They were stored from one to two weeks at room temperature in absolute acetone. At the time of injection for assay, groups of 10 to 20 pituitaries were homogenized in acetone, evaporated in vacuum, and resuspended in physiological saline solution. Assay animals, selected at 8½, 11½, 14½, and 17½ days of gestation, received intraperitoneally a single injection of 0.5 ml of pituitary suspension which contained the equivalent of one donor pituitary gland. The procedure for assay of gonadotropin (LH) by inducing ovulation in the pregnant female mouse has been described previously (Ladman and Runner, 1951a). Eighteen to 24 hours after injection, the number of tubal ova was determined. Six mice at each of the four stages of pregnancy were treated with the pituitaries from each of the five types of donor. This made possible a statistical evaluation of the data by the technique of multiple classification of analysis of variance (Snedecor, 1946).

Thirty-one pituitaries were collected for histologic study from the 5 donor stages of pregnancy. These glands were fixed in Helly's fluid, dehydrated and embedded in paraffin in the routine manner, and serially sectioned in the horizontal plane at 4 micra. Selected paraffin ribbons were chosen at varying distances through the gland and were stained by a modified trichrome technique developed for the mouse (Dickie, 1944). Differential cell counts were performed by the method of Rasmussen and Herrick (1922). Approximately 2000 cells were counted in each gland.

RESULTS

Weight of the pituitary gland during gestation. Fresh wet weights of 320 whole pituitaries collected from donor pregnant female mice during the gestation period are shown in Table 1 and Figure 2. The mean weight of pituitary glands at 6½ and 9½ days of pregnancy was 2.2 mg. Pituitary weight at 12½ days rose slightly to 2.3 mg. and by 15½ days a pronounced increase to 2.6 mg. was observed. The value at 15½ days was compared with the weights at 6½, 9½, and 12½ days and the differences were found to be statistically significant by the "t" test ($P = <0.01$). The mean pituitary

TABLE 1. WET WEIGHTS OF PITUITARY GLANDS FROM SWISS MICE DURING GESTATION

Day of pregnancy	Number of glands weighed	Mean weight of pituitary glands ± standard error (range of weights) mg.
6½	91	2.2±0.05 (1.0–3.8)
9½	66	2.2±0.05 (1.4–3.4)
12½	45	2.3±0.08 (1.5–3.8)
15½	41	2.6±0.09 (1.6–3.7)
18½	77	2.5±0.05 (1.6–3.7)

"t" Test comparisons of pituitary weights

6½ day vs. 15½ day: t=3.9; d.f.=130; P=<0.01
9½ day vs. 15½ day: t=4.1; d.f.=105; P=<0.01
12½ day vs. 15½ day: t=3.4; d.f.= 84; P=<0.01
6½ day vs. 18½ day: t=4.0; d.f.=166; P=<0.01
9½ day vs. 18½ day: t=4.2; d.f.=141; P=<0.01
12½ day vs. 18½ day: t=2.3; d.f.=120; P=<0.03
15½ day vs. 18½ day: t=1.3; d.f.=116; P=<0.20
9½ day vs. 12½ day: t=1.1; d.f.=109; P=<0.30

weight at 18½ days had dropped to 2.5 mg. but, when compared with the 6½ to 12½ day weights, the differences were still statistically significant. The difference between 15½ and 18½ day pituitary weights was found not to be significant. Differences of 0.2 mg. to 0.4 mg. in the mean weights of the pituitary glands were found to be statistically reliable.

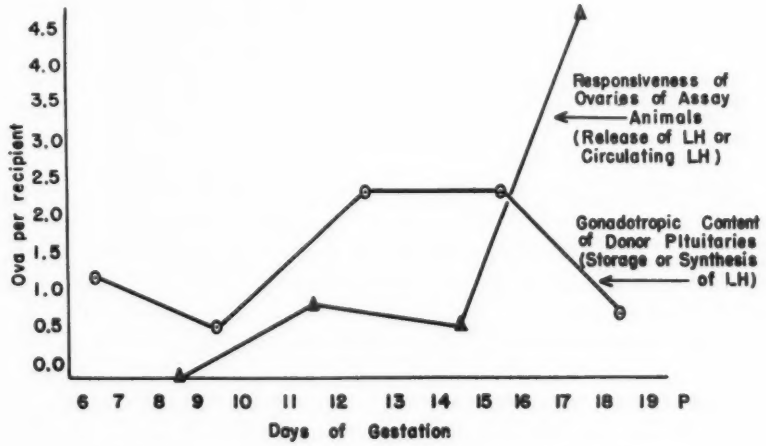

FIG. 1. Differentiation between storage and release of pituitary gonadotropin during gestation.

Bioassays of gonadotropin (LH) of pituitary glands. It should be re-emphasized that both donor and assay animals were pregnant mice distributed at selected intervals during the gestational period. This design of the experiment permitted tabulation of the number of released ova in the form of a latin square (Table 2).

TABLE 2. ASSAYS OF PITUITARY GONADOTROPIN DURING GESTATION*

	Days post coitum	Gestational stage of donors					Responsiveness of pregnant assay animals	
		6½	9½	12½	15½	18½	Total	(Ova per recipient)
Gestational	8½	0	0	0	0	0	0	(0.0)
Stage of Assay	11½	5	0	6	8	0	19	(0.6)
Recipients	14½	0	0	4	9	1	14	(0.5)
	17½	25	13	44	39	16	137	(4.6)
(Ova per recipient)	Total	30 (1.2)	13 (0.5)	54 (2.3)	56 (2.3)	17 (0.7)		
		Gonadotropic content of pituitary glands.						

Each square contains the sum of the ova produced by 6 assay animals following the administration of the equivalent of one pituitary gland to each animal.

* It is unfortunate that the numbers of ova in this study are small. Since pilot work had been done with older animals, the decreases in potency and response of younger animals were not anticipated.

The horizontal margin at the bottom of Table 2 summarizes the gonadotropic content of the pituitary gland during gestation in terms of the number of ova induced by donor pituitaries per assay recipient. At 6½ days, there was an appreciable amount of gonadotropin in the gland, as evidenced by the response of 1.2 ova per recipient. At 9½ days, this amount

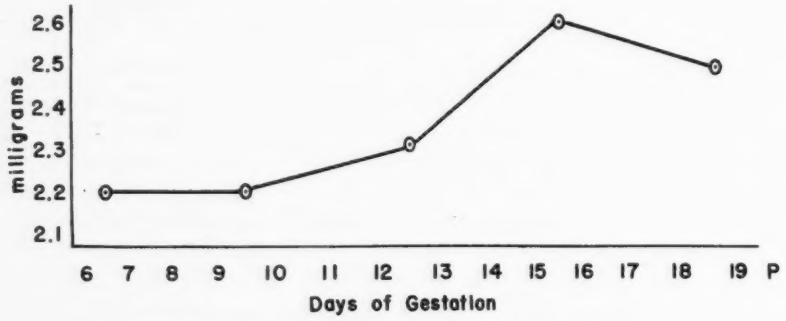

FIG. 2. Change of weight of pituitary glands during gestation.

had decreased substantially, resulting in the formation of only 0.5 ova per recipient, whereas, at $12\frac{1}{2}$ days, the pituitary content increased decidedly producing 2.3 ova per recipient. This level was maintained until $15\frac{1}{2}$ days. By $18\frac{1}{2}$ days, pituitary potency had decreased again so that only 0.7 ova per recipient were induced, which is appoximately the same level as at $9\frac{1}{2}$ days. As will be indicated later, these differences in gonadotropic content of pituitary glands during pregnancy were statistically significant ($P = < 0.04$).

Responsiveness of the assay animals at $8\frac{1}{2}$, $11\frac{1}{2}$, $14\frac{1}{2}$, and $17\frac{1}{2}$ days of gestation is summarized along the vertical margin at the right of Table 2. The assay in animals at $8\frac{1}{2}$ days of gestation did not ovulate, irrespective of the gestational stage of the pituitary substance injected. The response at $11\frac{1}{2}$ days and at $14\frac{1}{2}$ days of pregnancy was minimal, amounting to only 0.6 and 0.5 ova per recipient, respectively. The $17\frac{1}{2}$ day assay animals, on the other hand, showed a maximal response amounting to 4.6 ova per recipient. This increase in ovulatory response is particularly striking when it is realized that within a period of three days responsiveness of the pregnant animal increased almost ninefold.

Figure 1 summarizes the bioassays of pituitary glands shown in Table 2 by plotting the number of ova per recipient as a function of the stage in pregnancy. By this procedure, the number of ova induced at all stages of gestation and totaled in the vertical and horizontal axes of Table 2 are expressed in terms of storage (the content of donor pituitaries) and probable release (the responsiveness of assay animals' ovaries) of gonadotropin (LH) by the pituitary gland.

It is well known that ovulation is a sensitive indicator of circulating levels of LH. This ovulatory response to LH presupposes suitable preparation of the ovary by FSH. Dempsey (1937) has stated that FSH is presumably secreted at a constant rate throughout the estrous cycle. If it can be assumed that such constant ovarian preparedness exists during gestation (see Discussion), then alteration in the ovulatory response of assay animals should reveal circulating levels of LH and, therefore, the secretory activity of their own pituitary glands.

It is evident that the circulating levels of LH during pregnancy are low since the pregnant animal does not normally ovulate. However, upon administration of sufficient additional LH to augment the minimal level of circulating hormone, the ovary will be induced to ovulate, if it is capable of doing so at that time. This augmentation of the circulating level of LH in the pregnant mouse by injected pituitary glands of other pregnant mice, is, in essence, the basis not only for measuring the circulating level of the hormone but also for the interpretation of LH release. Thus, the observed changes in responsiveness signify that the period between $8\frac{1}{2}$ and $11\frac{1}{2}$ days

was accompanied by an increase in release of gonadotropin, and concomitant with this release, the content at $9\frac{1}{2}$ days was diminished. These results indicate that release exceeded synthesis. Between $11\frac{1}{2}$ and $14\frac{1}{2}$ days of gestation, the level of gonadotropin release seemed to be sustained. Yet, during the time interval between $9\frac{1}{2}$ and $12\frac{1}{2}$ days, the pituitary gland evinced a four-fold increase in potency suggesting that the rate of manufacture exceeded the rate of secretion. An extraordinary increase in release of gonadotropin appeared to occur between $14\frac{1}{2}$ and $17\frac{1}{2}$ days. This peak of secretion was accompanied by a three-fold decrease in content between $15\frac{1}{2}$ and $18\frac{1}{2}$ days of pregnancy, suggesting cessation of synthesis. The dynamic relationship between storage and release during this period seems adequately demonstrated.

Two surges of secretion of gonadotropin are indicated—one between $8\frac{1}{2}$ and $11\frac{1}{2}$ days of gestation and a more striking one between $14\frac{1}{2}$ and $17\frac{1}{2}$ days. Between $12\frac{1}{2}$ and $15\frac{1}{2}$ days, pituitary potency was maximal.

Statistical Analysis. The data from Table 2 were subjected to an analysis of variance in the manner presented in Table 3. Examination of column 5 of Table 3 indicates that the greatest source of variation was the responsiveness of the pregnant assay animals ($F = 23.8$; $P = <0.001$). Moreover, changes in the hormone content of the donor pituitaries at different times of pregnancy were reliably significant ($F = 3.0$; $P = <0.04$). The results of interaction between donor and assay groups as a source of variation were not critical.

TABLE 3. ANALYSIS OF VARIANCE FOR ASSAYS OF PITUITARY GONADOTROPIN DURING GESTATION

Source of variation	Degrees of freedom	Sum of squares	Mean square	F with probability	Per cent of total variation
Recipient assay animals (Responsiveness)	3	400.1	133.4	23.8 P = <0.001	82.0%
Donor animals (Potency)	4	68.0	17.0	3.0 P = <0.04	10.5%
Interaction	12	79.4	6.6	1.2 P = >0.05	4.1%
Subclass variation	100	561.7	5.6	—	3.4%
Totals	119	1109.2			

Further inspection of the statistical analysis of the assay data in Table 3 reveals a point of major significance. From examination of column 6 the 10% variation in pituitary potency observed during 5 stages of pregnancy seems relatively small. Nevertheless, this represents a shift of considerable magnitude when it is remembered that this small potency represents 82% of the variance in the responsiveness of assay animals. In other words, differences of 10% in the potency of the pituitaries were demonstrated to effect an increase in the responsiveness of assay animals by 82%.

Histologic observations. Since it had been shown by bioassay that during gestation the pituitary undergoes changes both in gonadotropic content

and in secretory rate, a histologic study of the anterior pituitary of pregnant mice was made in an attempt to corroborate the bioassay observations.

Four categories of cells were differentiated by a modified trichrome technique: acidophiles, basophiles, chromophobes and pycnotic cells. The percentages of these types at different stages of pregnancy are recorded in Table 4.

TABLE 4. DIFFERENTIAL CELL COUNTS OF THE ANTERIOR PITUITARY OF PREGNANT MICE

Days of pregnancy	Number of glands	Percentage of cells ± Standard error			
		Acidophiles	Basophiles	Chromophobes	Pycnotic
6½	6	28.7±2.3	1.3±0.3	65.6±1.8	4.4±0.8
9½	6	30.3±1.1	1.1±0.1	63.4±1.4	5.2±1.5
12½	6	33.5±2.3	0.7±0.2	61.8±3.0	4.1±1.6
15½	7	29.4±3.2	0.9±0.1	67.2±3.0	2.5±0.4
18½	6	31.1±1.7	1.1±0.1	64.2±1.6	3.6±1.0

Histologically, acidophiles exhibited a great diversity in the degree of granulation. There were not only large, spherical cells with densely packed acidophilic granules but also many small, granular cells during all the stages examined. Acidophiles appeared sparsely granulated at 6½ and 15½ days, and more fully granulated at 12½ and 18½ days. At 9½ days, the acidophiles were intermediate in granulation. Thus, acidophilic granulation seemed to reach a maximum between 6½ to 12½ days and again between 15½ and 18½ days, concomitant with increases in total acidophile percentages. Contrariwise, a notable decrease in granulation and number of acidophiles was observed between 12½ and 15½ days.

In the present study basophiles were identified as large cells that exhibited a distinctly blue cytoplasm and a well defined cell outline. With the technique used, no distinct granules occur in these cells, but only minute, dust-like particles. Since basophiles in the mouse are difficult to stain by most procedures, caution was observed in the identification and classification of these cells. Basophiles were located around the margin of the gland, in the centers of the lateral portions and in the isthmus. The percentage of the basophiles was lowest at 12½ days, amounting to 0.7%, and highest at 6½ and 18½ days, with values of 1.3% and 1.1%, respectively.

Chromophobes exhibited a variety of cellular forms ranging from cells with small nuclei and no observable cytoplasm to some with large vesicular nuclei and abundant cytoplasm. These latter cells were slightly colored with the anilin blue component of the trichrome stain, thus making the differentiation from basophiles somewhat difficult. These borderline cells were usually classified as chromophobes; a procedure which may account for the overall low percentage of recorded basophiles. The number of chro-

mophobes fluctuated inversely with the number of chromophiles; the largest increase in chromophobes occurred between 12½ and 15½ days from 61.8% to 67.2%.

Pycnotic cells were observed in all three cell types, although most frequently in the acidophiles. These pycnotic cells were characterized by their darkly staining and frequently indented nuclei. The chromatin was clumped, very dense and variably stained with either acid fuchsin or anilin blue. Chromophilic cells that were classified as pycnotic usually contained abundant clumped cytoplasmic granules. However, there were occasional cells in which distinct cell outlines and granules were lacking. These cells were considered to be pycnotic chromophobes. The pycnotic cells were usually confined to localized areas in the lateral parts of the anterior lobe. Total pycnotic forms varied between 2.5% at 15½ days and 5.2% at 9½ days.

The differences in cell frequencies in all categories in Table 4 were subjected to the "t" test and they were found not significant. It is quite evident from inspection of the standard error of each reading that there was a large variation in cell populations among animals in the same stage of pregnancy. This variability may be due to (1) genetic variation, for the Swiss mice used here were from pen-bred parents, (2) physiologic variation attributable to the number of embryos in utero, (3) alteration in other endocrine activities, or (4) variability of the areas studied consequent upon the difficulty of orientating small pituitaries for sectioning.

DISCUSSION

Bioassays. The amount of gonadotropic hormone in the anterior pituitary during stages of pregnancy has been reported for several species. There is wide variation between the results for different species, as well as some inconsistency within any one species. A number of European investigators (reviewed by Smith, 1939) have observed that the gonadotropic potency of pituitaries of pregnant women after the second month of gestation was very low. Bates, Riddle and Lahr (1935) found an increase of gonadotropic potency in the cow as pregnancy progressed, whereas Nalbandov and Casida (1940) reported a decline in potency of the pituitary gland in the same species from early to late pregnancy. Catchpole and Lyons (1934) and Hellbaum (1935) demonstrated that there was a rise and subsequent fall of pituitary gonadotropin during gestation in the mare. Wolfe (1931) suggested that the pituitary of the pregnant sow might undergo cyclic changes in hormonal output. The possibility of a cyclic change in pituitary gonadotropin during pregnancy was reaffirmed by Hill (1934) for rabbits. He observed that there was an initial decline followed by a rise and, during late pregnancy, a terminal fall. Evans and Simpson (1929) reported that the gonadotropic content of the pituitaries of pregnant rats was greater

than that of non-pregnant females. Recently, Zeiner (1952) has shown that the gonadotropic content of pituitaries of rats in the last trimester of pregnancy is higher than in earlier periods. He attributes this to storage of pituitary gonadotropin. Our own determinations of the gonadotropic content of pituitaries of pregnant mice show a cyclical change which coincides remarkably with the changes reported by Hill for rabbits.

One possible explanation for the inconsistencies between species are the variable differences in the activity of the placenta and pituitary during pregnancy. The data suggest that in the human and the mare the placenta may subserve more of the gonadotropic function than in the mouse, rat, rabbit and sow in which the production of gonadotropin may occur mainly in the pituitary. The high titre of circulating gonadotropic hormone of placental origin in the human and horse is a familiar feature of pregnancy. It should be noted that gonadotropin has been demonstrated in the placenta of the mouse (Mirskaia, 1930) and rat (Astwood and Greep, 1938). An important consideration is the fact that the circulating gonadotropin characteristic of all the above species subserves principally a luteotropic function. Present evidence does not indicate that the placental luteotropin which is indigenous to a species can evince FSH or LH like activities within that species.

The ovaries are apparently unresponsive during the first part of pregnancy. The $8\frac{1}{2}$ day recipients of our experimental series failed to ovulate, and previously Burdick and Whitney (1941) had shown that mice at 3 days of pregnancy are refractory to human chorionic gonadotropin (LH in activity in the mouse). This refractoriness of the ovaries may be associated with the inhibition of the action of the exogenous pituitary hormone by progesterone. In this connection, Atkinson and Hooker (1945) concluded that the progesterone titre of blood in the pregnant mouse is relatively high during the first half of gestation. Reduction in circulating progesterone and concomitant increase of estrogen began on about the tenth day. This shift in proportion of progesterone and estrogen should stimulate the output of LH, according to the "unit mechanism" theory (Fevold, Hisaw, and Greep, 1936; Fevold, 1944; Greep and Chester Jones, 1950). These factors would explain the responsiveness of the assay animals at $11\frac{1}{2}$ days. The great increase of responsiveness of the assay animals between $14\frac{1}{2}$ and $17\frac{1}{2}$ days would indicate that there was a further rise in release of hormone at that period.

Fluctuations in the circulating level of FSH might be evoked to explain the differences in ovarian responsiveness during pregnancy. In this connection, preliminary observations on follicular development to which the statistical techniques of Zuckerman (1951) were applied, indicated that the number of Graafian follicles varied during pregnancy but not significantly

(Ladman, 1952). Earlier, Evans and Swezy (1931) also reported variations in the numbers of Graafian follicles in pregnant rats. However, statistical analysis of their data casts doubt on their interpretation of "follicular cycles" in the pregnant rat (see Zuckerman, 1951). Therefore, if the numbers of Graafian follicles can be presumed to reflect the relative circulating amounts of FSH (Simpson, Li and Evans, 1951), then the data for the pregnant animal would appear to support Dempsey's (1937) observation that FSH is maintained at a relatively low and constant level throughout the normal estrous cycle, *i.e.*, at a time when the numbers of Graafian follicles are also known to vary, although not significantly (Evans and Swezy, 1931; Zuckerman, 1951). Thus, it may be justifiable to assume that FSH is relatively constant in the mouse during pregnancy. In our hypothesis, which explains the increased reactivity of assay animals' ovaries to exogenous mouse pituitary gonadotropin by an increased release of LH from the pituitary, the role of FSH upon the development of the follicles is acknowledged. The results of our assays were interpreted as representing essentially an augmentation effect of exogenous LH contained in the administered pituitary glands upon a postulated increase of LH released from the pituitary as pregnancy progressed.

The weights of the pituitary glands might support the observations on the content and the release of hormone (Fig. 2), if the alterations observed during pregnancy were assumed to be more closely related to the production of gonadotropin than to other functional activities of the gland. The mean pituitary weight changed little between $6\frac{1}{2}$ and $12\frac{1}{2}$ days, i.e., during a period of both active release and synthesis of gonadotropin. It is noteworthy that by $15\frac{1}{2}$ days the glands had become significantly heavier, although they failed to show increased potency, especially when compared with the $12\frac{1}{2}$ day value. If pituitary potency was expressed in terms of unit volume, a decrease in potency was observed between $12\frac{1}{2}$ and $18\frac{1}{2}$ days. The fact that assay values were similar at $12\frac{1}{2}$ and $15\frac{1}{2}$ days indicates the degree to which the increase of pituitary weight served to equalize the potency of the gland. By $18\frac{1}{2}$ days, pituitary weight decreased slightly. This occurred at a time when there was both massive release and, probably, diminished synthesis of gonadotropin.

Pituitary histology. Romeis (1940) reviewed the extensive histologic literature on the anterior pituitary as related to gonadotropic activity in many species under a variety of physiologic conditions. In reference to pituitary activity in the pregnant mouse, Urasov (1927) claimed that hypertrophied chromophobes which function as basophiles are characteristic of the pregnancy state. Agduhr (1932) observed that the acidophiles in mice showed the greatest increase in number during pregnancy, and Baer (1941–42) concluded that both acidophiles and basophiles were ac-

tively secreting at this time. None of the data of the above authors included differential cell counts of the anterior pituitary and, furthermore, the animals were not dated with respect to stages of pregnancy. The differences in histological techniques employed by these authors may be responsible for their varying conclusions. The inconclusive nature of our differential cell counts does not warrant a definite correlation of the alteration of cell types in the mouse pituitary during pregnancy with the bioassay results. Clarification of the cytology of the mouse's pituitary during pregnancy must await the application of further methods of fixation and of staining.

Many authors have ascribed the origin of LH to the acidophiles (Severinghaus, 1937; Dawson, 1946; Meyer, Biddulph and Finerty, 1946). Histologic data obtained during this study, especially with relation to the granulation of the acidophiles, seem to support this viewpoint. However, it is also known that pituitaries from gonadectomized animals contain large numbers of basophiles and yield bioassay responses indicative of large quantities of LH (Greep and Chester Jones, 1950). But histologic studies indicate that the basophiles of the mouse pituitary may respond differently than those of other species to gonadectomy (Woolley, Fekete and Little, 1941). From examination of human pituitaries employing a trichrome-PAS technique, Pearse (1952) has concluded that LH, being a glycoprotein, originates from cells giving a positive PAS reaction. Such PAS positive cells in the anterior pituitary appeared to be basophiles. Glycoprotein, as identified by the PAS reaction had been reported previously in the basophiles of the anterior pituitary of the rat (Catchpole, 1949).

In the present study it was observed that the decrease in number and granulation of acidophiles was coincident with an increase in responsiveness of the assay animals. These findings indicate that the circulating level of estrogen was high enough at $14\frac{1}{2}$ to $17\frac{1}{2}$ days to degranulate the acidophiles (Atkinson and Hooker, 1945) and suggest that, if LH originates from the acidophiles, it should be in circulating form at about $15\frac{1}{2}$ days. From these considerations it may follow that LH is released from the pituitary between $14\frac{1}{2}$ and $17\frac{1}{2}$ days, i.e., the time of maximal response of assay animals, and that the changes in acidophiles may be correlated with LH manufacture and release.

While observations on degranulation appear suggestive, the analysis of differential cell frequencies has more limited value. These results indicate: (1) that differential cell counts utilizing a trichrome staining method afford less information concerning the gonadotropic content and activity of the pituitary than a sensitive bioassay procedure, (2) that variability in pituitary histology in individual mice in the same stage of pregnancy may in part explain the inconsistent ovulatory responses obtained in some of the assay animals, and (3) that more precise methods of evaluating the cyto-

logic state of the mouse pituitary must be found before the histology can be relied on in the study of this species. In this respect, the application of Pearse's technique in the study of the mouse pituitary would be of great interest.

SUMMARY

Pituitary glands obtained from pregnant mice during five stages of gestation were assayed for gonadotropic potency by inducing ovulation in recipient pregnant mice. By this procedure gonadotropic potency was found to be maximal at 12½ and 15½ days of pregnancy, intermediate at 6½ days and minimal at 9½ and 18½ days, suggesting a cyclic fluctuation in gonadotropic content of the pituitary during pregnancy. Analysis of responsiveness of assay animals during four stages of pregnancy indicates that an increased rate of release of gonadotropic hormone occurs as pregnancy progresses. The gonadotropin which appears to be released from the pituitary at an augmented rate during the last half of pregnancy is considered to be LH.

Statistical analysis of the gonadotropic potency of the donor pituitaries as related to the responsiveness of recipient animals to gonadotropin demonstrates that a fluctuation of 10% in gonadotropic content can effect a variation of 82% in responsiveness of assay animals.

The weight of the pituitary gland increases significantly during pregnancy.

Histologic observations of the cell types of the anterior pituitaries of pregnant mice were made after staining with a modified trichrome technique. With this method, the variations in the percentages of cell frequencies at different stages of pregnancy were shown not to be statistically significant. It seems possible, however, that the transformation of acidophiles from a fully-granulated to a sparsely-granulated condition may be associated with the postulated release of LH from the pituitary during the last half of pregnancy.

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THE EFFECT OF PROGESTERONE ON PERSISTENT VAGINAL ESTRUS PRODUCED BY HYPOTHALAMIC LESIONS IN THE RAT[1]

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THERE is much evidence indicating that the central nervous system is of importance in the regulation of the hypophysial secretion of gonadotrophic hormones. (Anderson and Haymaker, 1948; Stieve, 1952). Present evidence favors the hypothesis that such control is mediated by neurohumors passing through the hypophysial portal system. (Harris, 1948, Markee, *et al.*, 1952), but the precise nature of such control has not as yet been clearly defined.

Although hypothalamic lesions have been employed for many years, systematic studies of pituitary function utilizing this technique have been infrequent. With the popularization of the Horsley-Clarke stereotaxic instrument by Ranson and his coworkers it has been possible to produce accurately localized lesions of varying size and thus to permit a more careful study of the problem.

Early workers frequently noted the occurrence of genital atrophy and, adiposity following hypothalamic damage (Erdheim, 1904; Bailey and Bremer, 1921) but considerable dispute existed whether this might be primarily due to hypophysial or diencephalic destruction. Smith (1927) clearly showed that the adiposity was not due to pituitary insufficiency but could easily be produced by hypothalamic lesions. He reported that genital atrophy could also be induced by hypothalamic lesions without damage to the pituitary.

Smith also mentioned that hypothalamic damage sometimes resulted in prolonged vaginal estrus in the rat (1927), but this was first extensively studied by Dey and coworkers. Large, bilateral lesions in the anterior hypothalamus produced constant estrus in the guinea pig and this was shown to be associated with follicular, cystic ovaries, without corpora lutea, and with hypertrophied uteri. Lesions at the junction of the pituitary stalk produced atrophic genitalia and constant diestrus while posterior

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hypothalamic lesions had no particular genital effect. (Dey, 1943a). The anterior hypothalamic lesions abolished the mating behavior of the animals. (Brookhart, Dey, and Ranson, 1941). Ovulation and corpus luteum formation could not be induced by either copper sulfate or acetylcholine injection in the persistent estrus animals. (Dey, 1943b). Large lesions directly in the pituitary did not reproduce this phenomenon and had no effect on the reproductive organs even though destroying 2/3 of the hypophysis unless the median eminence were also damaged. (Dey, Leininger, and Ranson, 1942).

Apparently no further investigation with this technique was made until Hillarp (1949) extended the procedure to the rat and found that by making small, bilateral, superficial lesions between the paraventricular nucleus and hypophysial stalk constant estrus could be produced. The ovaries were similar to those found by Dey in the guinea pig, although no details were given of uterine or ovarian size.

The present investigation was undertaken to extend the observations of the effect of hypothalamic lesions on reproductive activity in the rat. It was found that in those animals in which persistent estrus was produced, cyclic ovarian activity and endogenous progesterone production could be induced by the daily administration of small doses of progesterone.

EXPERIMENTAL

Female albino rats of the Harvard, Charles River, Sprague-Dawley and Holtzman strains were utilized. Bilateral electrolytic lesions were made in the hypothalamus as previously described (Greer, 1951), using a modification of the stereotaxic instrument devised by Krieg for the rat. (Krieg, 1946). A total of 197 young adult animals weighing 225–275 gm. were utilized. Lesions were made 1 mm. on either side of the midline and from 0 to 1.5 mm. above the floor of the skull. The lesions varied in the rostrocaudal plane from 5 to 7.2 mm. anterior to the external auditory meatus. A direct current of from 1 to 8 ma. for 15–30 second was used. Electrodes of varying size and material were employed, but it was found that a common, round, steel sewing needle with the point cut off and coated with insulating varnish, except for the tip, gave the best performance. Once the technique had been mastered, 6–8 animals per hour could easily be operated on with a negligible operative mortality.

Unilateral ovariectomy was performed 2 weeks before beginning progesterone injections in 7 animals in order to compare ovarian structure before and after progesterone.

Vaginal smears were made at the same time each day, either from 2–3 P.M., or 4–5 P.M. in the various groups. A small vaginal spatula or glass pipette was used. In the initial experiments, smears were made daily for

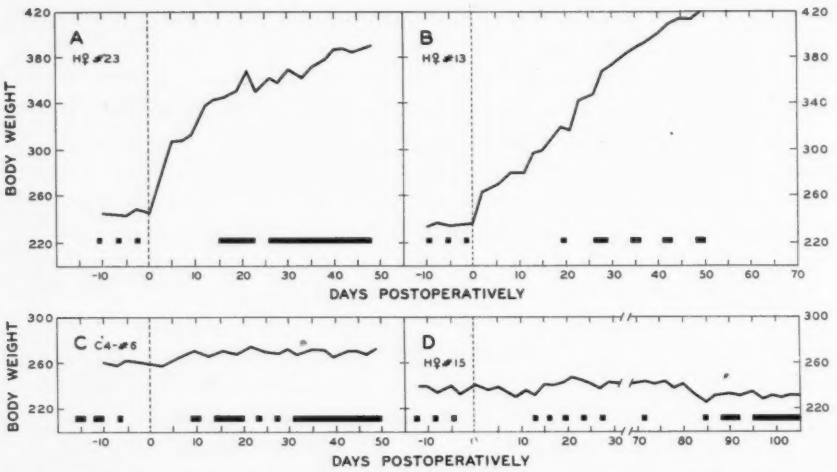

FIG. 1. Variations in the development of persistent vaginal estrus. The solid black bars represent periods of cornified vaginal smears. A) Obesity and persistent estrus. B) Obesity with relatively normal cycles. C) Persistent estrus without obesity. D) Delayed onset of persistent estrus.

2 weeks preoperatively and were continued until the animals died or were killed. Only animals having normal preoperative cycles were used. In some of the later experiments in which it was desired to utilize only the animals in persistent estrus, smears were not begun until 2–3 weeks post-operatively.

In one set of experiments, the animals were fed 0.06% propylthiouracil in ground Derwood pellets for 2 weeks in order to ascertain whether the lesions responsible for persistent estrus were the same as those which prevented the goitrogenic response to antithyroid drugs (Greer, 1951). In all other experiments, the animals were fed either Purina fox checkers or Derwood pellets.

Animals were weighed every 2 days beginning 2 weeks or more preoperatively in the initial experiments. In later experiments the animals were weighed at operation and approximately once weekly after that.

Tissues were fixed in formalin or Zenker's fluid. The heads were prepared at autopsy by trimming away all skin, the lower jaw, and the bony dorsum of the skull and leaving the remainder in formalin for one week or longer. At that time the brain was removed and the hypothalamic area serially sectioned at 10 μ, every tenth section being mounted. Similar serial sections made of the ovaries and pituitary. Uteri, thyroids, and adrenals were also sectioned at 10 μ. The brains were stained with 2% toluidine blue for 6 hours. All other tissues were stained with hematoxylin and eosin.

The progesterone preparation used (*Proluton*, Schering) contained 5 mg. dissolved in each cc. of corn oil solvent. All injections were given subcutaneously once daily at rotated sites.

RESULTS

Onset of Persistent Estrus

In confirmation of Dey and Hillarp, anterior hypothalamic lesions were found to produce persistent vaginal estrus. In the initial experiments 13 of 17 animals with such lesions developed persistent estrus. In all but one case a period of 7–15 days of vaginal diestrus followed the operation. This did not seem to be pseudopregnancy since in 5 animals deciduomata could

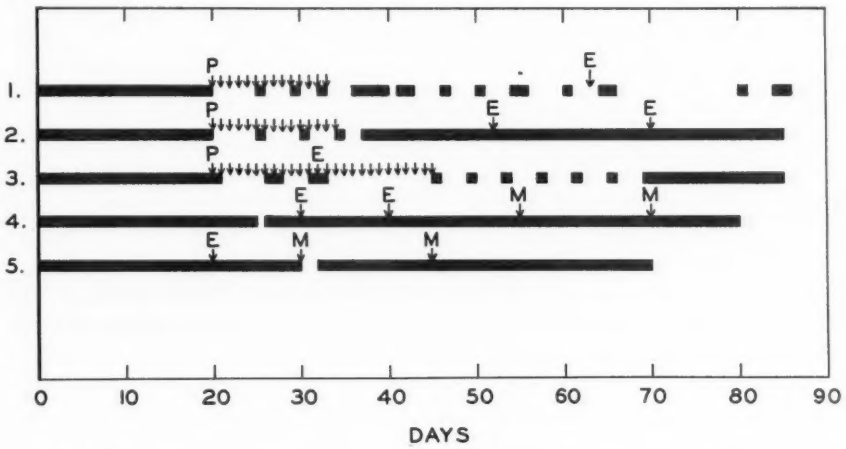

FIG. 2. Illustration of types of response to procedures in persistent estrus. P = progesterone injection; E = electrical stimulation of cervix; M = male placed in cage overnight. 1) Progesterone causes resumption of normal cycles which persist following cessation of injections. Cervical stimulation followed by pseudopregnant interval during post-progesterone spontaneous cycles. 2) Progesterone cycles followed by resumption of persistent estrus. Cervical stimulation has no effect during persistent estrus. 3) Cervical stimulation causes pseudopregnancy during progesterone cycles. Post-progesterone normal cycles followed by resumption of persistent estrus. 4&5) Cervical stimulation and the presence of a male in the cage have no effect on vaginal smear except for one short period of diestrus.

not be produced by scratching the endometrium 4–5 days postoperatively. Following this diestrus interval, 7 of the rats then had continuous vaginal cornification. In one instance, there were prolonged periods (3–5 days) of estrus alternating with diestrus intervals of approximately equal length.

Although once persistent estrus had appeared it usually remained indefinitely, occasionally 1 or 2 days of diestrus would be interspersed at irregular intervals during the period of observation.

In one rat persistent estrus began immediately postoperatively. Four rats had several normal post-operative cycles preceding the constant estrus. One animal had normal cycles for three months after the hypothalamic lesions were produced, then went into constant estrus.

Twenty of the rats in the later experiments had persistent vaginal cornification, making a total of 33 such animals.

Unilateral Ovariectomy

In 7 persistent estrus animals, the left ovary was removed and the smear observed for at least 2 weeks before progesterone treatment was begun. In all instances continuous cornification was maintained in spite of a 50% reduction of ovarian tissue.

Relation of Persistent Estrus to Obesity

In confirmation of the work of other investigators, most of the animals with lesions in the vicinity of the ventromedian nucleus developed obesity. (Brobeck, 1946). The area responsible for the obesity was apparently closely related to the area controlling the estrous cycle, since most of the rats with persistent estrus showed a gain in weight of 20–50 gm. and a few gained more than 200 gm. However, the areas seemed to be independent since marked obesity occurred in animals with normal cycles or prolonged diestrus and persistent estrus occurred in 5 which had no gain in weight.

Those animals which gained weight began to do so immediately postoperatively and this had no relation to the time at which persistent estrus developed.

Ovarian and Uterine Weight and Structure

The ovaries of animals which had been in persistent estrus for 2–3 weeks or longer had a characteristic appearance. No newly-formed corpora lutea were seen and only a few, old, atretic ones could be found. Many

TABLE 1. COMPARISON OF BODY AND ORGAN WEIGHTS OF ANIMALS WITH HYPOTHALAMIC LESIONS. THOSE WITH PERSISTENT ESTRUS HAD RECEIVED AT LEAST ONE COURSE OF PROGESTERONE BEFORE AUTOPSY

	Number animals	Body wt. gm.	Thyroid mg.	Ovaries mg.	Adrenals mg.	Uterus mg.
Normal Cycles	6	304 ± 36*	16.9 ± 1.7	58.9 ± 6.6	61.1 ± 5.8	602 ± 69
Persistent Estrus	10	335 ± 21	19.1 ± 0.9	41.4 ± 3.7	63.9 ± 6.2	652.5 ± 64

* Standard Error.

TABLE 2. COMPARISON OF BODY AND ORGAN WEIGHTS OF ANIMALS WITH HYPOTHALAMIC LESIONS FED 0.06% PROPYLTHIOURACIL FOR 2 WEEKS

	Number animals	Body wt. gm.	Thyroid mg.	Ovaries mg.
Normal Cycles	18	269 ± 5*	37.5 ± 2.2	53.1 ± 2.4
Persistent Estrus	6	262 ± 23	34.3 ± 5.7	36.7 ± 3.4

* Standard Error.

follicles, in various stages of development, were present, some having very large antra. There were well developed interstitial glands. These findings are in agreement with previous observations of Dey in the guinea pig and Hillarp in the rat.

The mean ovarian weight was ⅓ less in the persistent estrus animals treated with progesterone than in control animals with hypothalamic lesions which had normal estrous cycles. (Table 1). In the experiments in which all animals received propylthiouracil for 2 weeks and none received progesterone, the ovaries of those with persistent estrus also were ⅓ lighter than the ovaries of those with normal cycles. (Table 2).

Ovarian weights of the 7 persistent estrus rats unilaterally ovariectomized before progesterone treatment was begun were approximately doubled at the end of the experimental period. (Progesterone plus deciduoma induction. See Table 3.)

No significant difference in weight or microscopic appearance was noted between the uteri of persistent-estrous animals which had received progesterone and those with normal cycles.

Relation of Persistent Estrus to Other Endocrine Organs

There was no significant difference in the weight or microscopic appearance of thyroid and adrenals of the animals having normal cycles and those with persistent estrus. These organs were also not different from those of untreated control animals.

Twenty-four rats with anterior hypothalamic lesions, 6 with persistent

TABLE 3. BODY AND ORGAN WEIGHTS OF ANIMALS WITH PERSISTENT ESTRUS FOLLOWING HYPOTHALAMIC LESIONS. ALL ANIMALS WERE GIVEN COURSE OF PROGESTERONE AND DECIDUOMATA WERE PRODUCED, DURING TREATMENT. AUTOPSY ON EIGHTH DAY OF PSEUDOPREGNANCY, 4 DAYS AFTER PUTTING THREAD THROUGH UTERUS. LEFT OVARY REMOVED 2 WEEKS BEFORE PROGESTERONE TREATMENT BEGAN

Number animals	Body wt. mg.	Left ovary mg.	Right ovary mg.	Uterus mg.
7	338 ± 18*	24.9 ± 3.6	51.2 ± 5.6	1176.74

* Standard Error.

estrus and 18 with normal cycles, were fed a diet of 0.06% propylthiouracil for 2 weeks to test their ability to secrete thyrotropin. Anterior hypothalamic lesions previously have been shown to prevent the goitrogenic response to thiouracil feeding (Greer, 1951). Four animals with normal cycles and one with persistent estrus did not exhibit the usual thyroid hypertrophy on this regimen, so that the hypothalamic area essential for normal estrous cycles is presumably not the same as that necessary for the goitrogenic response to thiouracil.

The hypophyses of all animals with hypothalamic lesions appeared normal except for those of 3 animals with normal cycles and one with persistent estrus each of which had small infarcts in one lobe.

Mating Behavior

At no time was sexual receptivity observed in rats with persistent vaginal estrus. Although 12 such animals were placed with males for 1–5 days, both during the period of constant estrus and when normal vaginal cycles had been resumed during progesterone administration, copulation was never observed and spermatozoa were never found in the vaginal smears. Occasionally, however, 1–2 days of diestrus were induced after a male was placed in the cage overnight.

Results of Cervical Stimulation During Persistent Estrus

In 9 animals a strong faradic current was applied to the cervix for 15–40 seconds. Although this invariably produced clonic convulsions of the hind legs, it produced no change in the estrous vaginal smear.

Effect of Progesterone Injections on Persistent Estrus

Because of the observation of Everett (1940) that daily injections of 0.5–1.0 mg. of progesterone in oil would produce a resumption of ovulation and normal cycles in a strain of rats with spontaneously occurring persistent estrus, it was decided to investigate the effect of progesterone in the hypothalamic animals. The progesterone seemed to have an identical action in rats with hypothalamic or spontaneous constant estrus.

a) *A single injection* of 0.5 mg. progesterone in 3 persistent-estrous animals was followed by diestrus smears in 24–48 hours. Diestrus lasted about 48 hours; persistent vaginal cornification was then resumed.

b) *Small daily injections* of progesterone invariably produced a resumption of vaginal cycles with fairly regular 4–6 day intervals. Normal cycles could also be induced in those animals which had alternating periods of prolonged estrus and diestrus. Although 0.5 mg. progesterone was usually sufficient, occasionally 1.0 mg. daily was required in the more obese animals.

c) *Cervical stimulation* with a faradic current during the period of daily progesterone administration usually resulted in a prolonged diestrous interval of 8–12 days even though the progesterone injections were maintained. Since deciduomata could be produced by passing a thread through the uterus on the fourth day of this diestrous interval in 7 unilaterally ovariectomized animals, it was evident that pseudopregnancy had been produced. (Table 3).

d) *Large corpora lutea* were formed in the ovaries during the period of progesterone administration. These were histologically similar to those found in normal ovaries except that some ovaries contained several lutein cysts, apparently the result of luteinization of some of the pre-progesterone cystic follicles.

Effect of Stopping Daily Progesterone Injections

Progesterone was given daily until several normal cycles had been produced, at which time it was withdrawn. In about 50% of the animals this was followed by a resumption of persistent estrus within 48 hours. In the other 50%, normal vaginal cycles were maintained indefinitely after one or more courses of progesterone. In one animal, persistent estrus was resumed after 2 months of normal cycles without progesterone.

Occurrence of Prolonged Diestrus

Although many lesions were produced, ranging from the optic chiasm to the mammillary bodies, persistent diestrus was never observed. In 7 of the 197 animals, periods of extremely prolonged diestrus occurred, but this was interrupted at one or more irregular intervals by an estrous smear. All but two of these animals died before they could be autopsied. The remaining two had thyroids, adrenals, ovaries and uteri indistinguishable from those of normal controls in weight and microscopic appearance except that one had an encapsulated, multiocular, 7×15 mm. endometrial cyst in the right horn of a dilated hydrometrium. One ovary was removed from one other animal in this group before it died and was also found to be normal.

Daily injections of 0.5 mg. progesterone following unilateral ovariectomy had no effect on the prolonged diestrus of these rats or on the weight or structure of their ovaries.

Effect of Progesterone on Vaginal Cycles and Uteri of Normal Rats

In order to ascertain that the 0.5 mg. progesterone given daily in these experiments was below the minimal requirement for a direct effect on uterus and vagina, seven normally cycling rats were given this regimen daily for 16 days. On the twelfth day a thread was passed through one uterine horn

and on the last day the animals were autopsied. There was no discernible effect on ovaries, vaginal cycles, or uterus and no deciduomata were produced.

Location of Lesions Causing Persistent Estrus

Hillarp has stated that persistent estrus can regularly be produced by superficial bilateral, hypothalamic lesions between the paraventricular nucleus and hypophysial stalk. The lesions in the persistent-estrous animals of this investigation are consistent with this statement since almost all were ventral to the paraventricular nuclei and slightly rostrad or caudad to it, if not in the same plane. Many animals which had normal cycles had lesions in this same area, however.

Endocrine abnormalities were never seen in animals with lesions in the posterior hypothalamus. Although much of the mammillary body was destroyed in some animals, they maintained normal estrous cycles.

The lesions varied in size from those slighty larger than the needle track to those destroying a considerable portion of the central hypothalamus. In 5 animals the electrode was passed to the base of the brain and withdrawn, using the same co-ordinates on the Krieg machine as were found to most consistently produce constant estrus, but without passing an electric current. Persistent estrus was not produced in any by this procedure.

In the one animal with prolonged diestrus in which it was possible to obtain hypothalamic sections, the lesions were between the paraventricular and preoptic nuclei. This location is similar to that found by Hillarp in animals with prolonged diestrus.

DISCUSSION

The earlier investigations of Dey and of Hillarp were readily confirmed, although no animal was produced which had persistent diestrus and marked genital atrophy. Most probably no lesions in this investigation involved the same anatomical location, i.e., the median eminence, which Dey found would result in genital atrophy. It is of interest that Hillarp also did not report marked genital atrophy to follow any of his lesions in the rat. The prolonged diestrus which he observed following lesions anterior to the paraventricular nucleus was associated with ovaries and uteri of normal size and histologic appearance. Similar findings were made in this investigation.

Although the development of persistent estrus was usually accompanied by some degree of obesity, it is quite unlikely that the obesity was responsible for the development of the changes in the sexual cycle. Female rats have been force-fed in this laboratory until they attained a weight in excess

of 700 gm. No change was ever produced in the vaginal cycle other than a slight prolongation of the diestrus interval in some (Greer and Payne, 1949).

The association of obesity and menstrual disturbances, particularly amenorrhea, is frequently seen clinically. It is possible that this association is due to some disturbance in hypothalamic function. That this may be so is indicated by preliminary studies of 7 obese young women with secondary amenorrhea in whom apparently normal, cyclic bleeding was induced by the *continuous*, daily administration of 10 mg. oral pregneninolone (Greer, 1952).

It is not clear at present why anterior hypothalamic lesions should cause persistent estrus. Dey and Hillarp postulated that the lesions interfered with the secretion of LH and that chiefly FSH plus a small amount of LH was released by the pituitary, accounting for normal follicular development and estrogen secretion. Dey postulated that LH could not be secreted in adequate amounts since the injection of acetylcholine or copper sulfate did not produce ovulation in hypothalamic persistent-estrous animals as it did in normal animals.

Whatever the mechanism for ovarian luteinization and progesterone secretion may be, it is apparently restored by the daily injection of small doses of progesterone. In this respect hypothalamic and spontaneous ersistent estrus are quite similar. How progesterone accomplishes this action is at present unknown. Since normal cycles could be maintained in approximately one half the persistent-estrous animals following one or more courses of progesterone, it may have a "priming" effect in the hypothalamic animals. Endogenous secretion may become adequate to maintain normal cyclic function. It is also possible that hypothalamic reorganization takes place after a time and that the animals would have had a return of normal cycles without progesterone treatment. Although a spontaneous return of normal cycles was never observed in persistent-estrous animals followed for as long as 10 months, most of the animals received at least one course of progesterone during that time. In one animal, however, a course of progesterone was followed by a maintenance of normal cycles for 2 months, following which presistent estrus reappeared. This indicates that permanent repair of the underlying defect may not have occurred.

Not only could normal cycles and corpus luteum formation be induced by progesterone administration in the persistent-estrous animals, but pseudopregnancy and a marked increase in the secretion of endogenous progesterone could be caused by cervical stimulation during the period of progesterone treatment. Thus the ability of nervous stimuli to alter cyclic gonadotrophic function was not destroyed by the hypothalamic lesions.

SUMMARY

Bilateral hypothalamic lesions ranging from the optic chiasm to the mammillary bodies were produced stereotaxically in 197 young adult female rats. Lesions in the posterior hypothalamus had no effect on the sexual cycles. Of the animals with anterior hypothalamic lesions, 33 entered persistent vaginal estrus, 7 had prolonged diestrus, and 7 had alternating periods of prolonged estrus and diestrus.

Daily administration of 0.5 mg. progesterone to persistent-estrous animals was followed by a resumption of fairly normal vaginal cycles and corpus luteum formation. Pseudopregnancy could be induced by cervical stimulation in the persistent-estrous animals during the periods that they had progesterone-induced vaginal cycles but not while they had continuous vaginal cornification. Progesterone, in the dosage administered, had no effect on hypothalamic animals with prolonged diestrus or on normal animals, as measured by the vaginal and uterine response.

The mechanism of action of progesterone in inducing normal cyclic behavior in lesioned animals is unknown, but the phenomenon indicates that the changes in the reproductive cycle following hypothalamic lesions are of a secondary nature and that the primary cyclic mechanism is capable of being reactivated.

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THE RESPONSE OF THE PUBIC SYMPHYSIS OF THE MOUSE TO EXTRACTS OF PREGNANT RABBIT SERUM AND PREGNANT SOW OVARIES AND ITS APPLICATION AS AN ASSAY METHOD[1]

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GARDNER reported in 1935 that the pubic symphysis of the mouse separates during pregnancy and that a small amount of separation could be obtained by prolonged treatment of castracted mice with estrogens (Gardner, 1936). Hall and Newton (1946, 1947), however, showed that it was necessary to inject castrated mice with pregnant rabbit serum (PRS) in addition to a preliminary treatment with estrone in order to duplicate the rapid rate of separation observed during pregnancy. Further work by Hall (1948) indicated the necessity of pretreating the mice with estrogen for a period of five to eight days in order to obtain the maximum response to PRS.

Because PRS is an excellent source of relaxin, and since the same type of response, i.e. pelvic relaxation, is obtained in both the mouse and guinea pig, Hall (1948) suggested that the measurement of pubic relaxation in the mouse could be used as an assay for relaxin. This would obviate the subjectivity present in the guinea pig palpation assay. Recently, however, relaxin was defined in terms of its effect on the guinea pig (Frieden and Hisaw, 1952) and since highly purified extracts of pregnant sow ovaries (PSO) do not seem to act similarly in the mouse and the guinea pig, we have avoided referring to the hormone effecting the separation of the mouse symphysis as relaxin. Whether relaxation of the pubic symphysis of the mouse and guinea pig is caused by the same substance is yet unknown. It is the purpose of this paper to describe the methods and conditions for the quantitative measurement of the separation of the mouse

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pubic symphysis, and to define a unit of response for this phenomenon in the mouse. Extracts from both the serum of pregnant rabbits and the ovaries of pregnant sows were used in this investigation.

MATERIALS AND METHODS

Sexually mature mice of the Swiss BAG albino strain were used in this study. The animals were injected daily for seven days with 1 μg. of estradiol in 0.05 ml. of sesame oil given subcutaneously. All X-ray photographs were taken with a Westinghouse dental X-ray machine, and Kodak DF-7 dental X-ray films were used. The mice were anaesthetized with 2.5 mg. of sodium nembutal (sodium pentobarbital) injected intraperitoneally in 0.2 ml. of 1% saline, and were then placed in a prone position, the pelvis being immobilized against the X-ray film by means of a loose rubber band (Fig. 1). The cone of the machine was placed directly beneath the tail at an angle of 38° with respect to the X-ray film. A 145 kilovolt peak, a 15 milliampere current, and a time

FIG. 1. An anesthetized mouse in proper position for X-ray photography of the pubic symphysis.

exposure of $\frac{3}{4}$ second were used to expose the films. Each X-ray film was identified by imprinting a code number and date on the outer wrapper with an ordinary graphite pencil. These markings appeared on the developed films, thus providing a permanent record for each X-ray. The films were developed according to routine procedure and the degree of relaxation of the pubic symphysis was measured to the nearest 0.1 mm. by projection onto millimeter ruled graph paper at a constant magnification of 10 diameters.

RESULTS

Minimal Positive Response

During the course of this investigation, X-ray photographs were taken of the pelves of estrogen-treated mice and groups of mice receiving inef-

TABLE 1. DATA SHOWING THE MAXIMAL RESPONSE OBTAINED WITH ESTROGEN ALONE OR ESTROGEN + INACTIVE PROTEIN EXTRACTS IN CASTRATED AND INTACT MICE*

No. of mice	Treatment	Mean response in mm. ± S.E.
	A. Castrate mice	
4	Estrogen alone	0.10 ± 0.043
4	Estrogen alone	0.02 ± 0.063
4	Estrogen alone	0.02 ± 0.076
3	Extract of rat placenta: 70 mg.	0.00 ± 0.00
5	0.1 mg. PRS	0.06 ± 0.026
5	0.1 mg. PRS	0.10 ± 0.020
Total 25		0.03 ± 0.017
	B. Intact mice	
4	Sow ovary discard: 100 mg.	0.07 ± 0.014
3	Mouse placenta discard: 100 mg.	0.03 ± 0.024
9	Cysteine inactivated relaxin: 0.44 mg.	0.03 ± 0.047
Total 16		0.04 ± 0.028

* All animals were primed with 1 μg. estradiol daily for 7 to 10 days and roentgenograms of the symphysis pubis were taken immediately before, and 24 hours after the injection of the test preparation. Response is recorded as the difference between these two measurements of the pubic gap. Per cent relaxed is based upon the per cent of mice showing responses of 0.30 mm. or over. None of the above mice were relaxed.

fectual amounts of various protein extracts in addition to the estrogen (Table 1). These experiments were carried out at different times over a period of 18 months and the data were used to determine (1) the effect of estrogen priming on pubic separation (2) the error involved in the technique of making the measurement, and (3) the difference if any between the castrated and intact mouse. In all instances the animals were primed with 1 μg. of estradiol daily for 7–10 days. No marked differences were observed between any of these groups, and in no group did the average increase in pubic gap exceed 0.10 mm. during a 24-hour period. Furthermore, the combined average increments for the castrated and the normal mature mice were 0.03 mm. and 0.04 mm. respectively; this difference is not statistically significant. Thus, in so far as the effects of estrogen priming are concerned, normal mice can be used as readily as castrated mice for the assay of the relaxative hormone.

A significant, positive response is considered to occur when the probability that it could be due to estrogen priming or variation in technique is no more than one chance in one hundred. Assuming that the pubic separation in the control animals would conform to a normal distribution curve, it is possible to determine the separation which would occur by chance in 1 per cent of the population by multiplying the standard error of the control groups by the corresponding value for 1 per cent t. This value is 0.24 mm. for the castrated group and 0.32 mm. for the normal

mice. For convenience in measurement, an increment of 0.30 mm. in 24 hours is the minimal response which we consider to be significant. On this basis, none of the 41 animals in Table 1 were relaxed. In data to be cited, the per cent of animals considered to have undergone relaxation is calculated on the basis of this minimal significant amount of pubic relaxation.

Time required for relaxation

Ten normal mice were primed with 1 μg. estradiol daily for 7 days and given 10 mg. of a crude 5 per cent saline extract of pregnant sow ovaries (Preparation S-1) 24 hours after the last injection of estrogen. X-ray photographs were taken at 8, 16, 24 and 36 hours after the injection of the extract. Seven of the ten animals were significantly relaxed and showed an

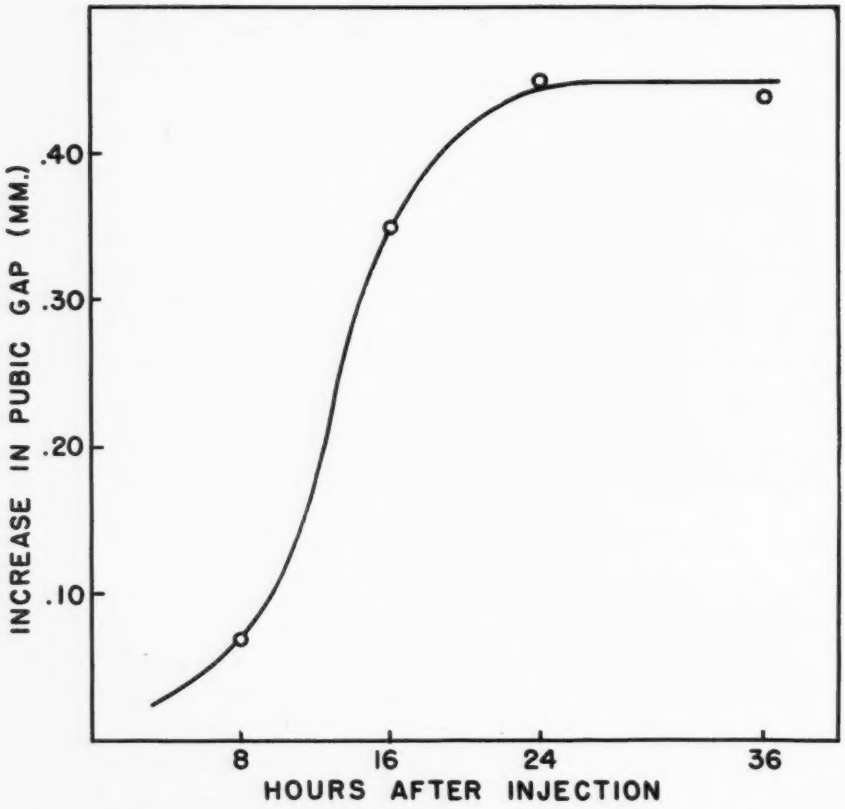

FIG. 2. Pubic separation as a function of time following a single 10 mg. dose of sow ovary extract S-1.

average increase in the pubic gap of 0.07, 0.36, 0.45, and 0.44 mm. respectively from the original separation. The relaxation curve (Figure 2) is drawn on the basis of these animals only. It is apparent that relaxation begins after 8 hours and is practically complete before 24 hours.

Effects of prolonged priming

Fourteen castrated mice were treated with 1 μg. estradiol daily. On the 8th day, one group of six mice received 20 mg. each of a crude extract of pregnant rabbit serum. The remaining eight mice were continued on estradiol and X-ray photographs were taken daily from the 9th to the 13th day and every other day thereafter. This second group of mice received 20 mg. of the same preparation of PRS on the 23rd day of estrogen treatment. The results are shown in Figure 3.

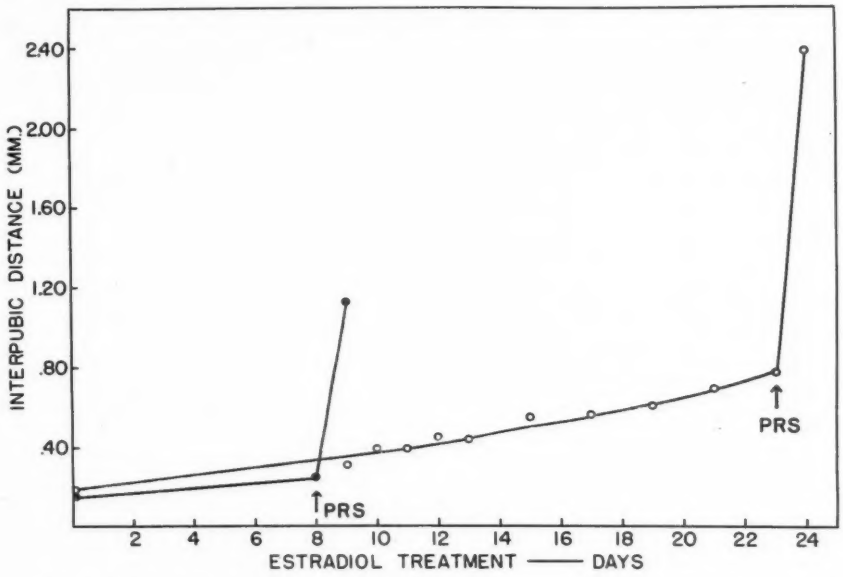

FIG. 3. Separation of the pubic symphysis of the mouse during estrogen treatment for 8 and 23 days and the response to a standard dose of pregnant rabbit serum extract (PRS) following the estrogen treatment.

Estrogen caused a slow increase in the separation of the pubic bones which may be contrasted with the more extensive and rapid effect of a single injection of the relaxative hormone. An increased sensitivity to the hormone was observed for the mice receiving prolonged treatment with estradiol. The effect of a single dose was increased from a response of 0.88 ± 0.18 mm. (Mean $\pm$ S.E.) after 8 days of estrogen priming to one of

1.61 ± 0.17 mm. for the 23-day period. All of the animals showed significant separation of the pubes, which is an indication that sufficient material was given to obtain a maximal response. Statistical analysis verified that the separations due to PRS and the differences between the increments recorded on the 9th and 24th day are highly significant (P values were less than 0.001).

The assay of PRS and PSO

An extract of pregnant rabbit serum (PRS) was prepared by the method of Albert and Money (1946), and assayed by the present technique. Forty-six castrated mice were primed for seven days with daily, subcutaneous injections of 1 μg. estradiol. After control X-ray photographs were taken on the 7th day, the mice were placed randomly into six groups and each mouse was injected subcutaneously with either 1, 2, 5, 10, 20 or 40 mg. of the PRS preparation. Roentgenographic measurements were made on the 8th day; the injections of both estrogen and PRS were repeated, and X-ray photographs were again taken 24 hours later. Thus, the possibilities of both a single and double dose assay were examined. The results are summarized in Table 2 and in Figures 4 and 5.

It can be seen from the data that, as the dose of PRS is increased, a greater percentage of the mice show significant separations of the pubic

TABLE 2. ASSAY OF PREGNANT RABBIT SERUM EXTRACT (PRS)

Number of mice	Total dosage (Mg.)	Per cent relaxed	Pubic separation mm. ±S.E.	Standard deviation
		A. Single dose		
5	1.0	20	0.16 ± 0.04	± 0.09
10	2.0	20	0.11 ± 0.05	± 0.15
10	5.0	50	0.40 ± 0.11	± 0.30
8	10.0	75	0.91 ± 0.25	± 0.71
9	20.0	100	1.17 ± 0.20	± 0.61
4	40.0	100	1.15 ± 0.42	± 0.85
		B. Double dose		
5	2.0	80	0.40 ± 0.11	± 0.45
10	4.0	60	0.31 ± 0.06	± 0.17
10	10.0	100	0.67 ± 0.07	± 0.23
8	20.0	88	0.46 ± 0.41	± 1.15
9	40.0	100	1.64 ± 0.19	± 0.57
4	80.0	100	1.70 ± 0.61	± 1.22

For single dose:
Equation of line, $y = a + bx$,
b = slope of line between doses of 2 and 20 mg.

s = standard deviation; derived from $\sqrt{\frac{\Sigma(y - Y \text{ calc})^2}{n' - 2}}$ where Y calc is the value of Y calculated from the equation of the line and n' is the number of doses.

λ is the error of x as estimated from y and is equal to s/b.

$b = 1.09$
$s = 0.100$
$\lambda = 0.092$

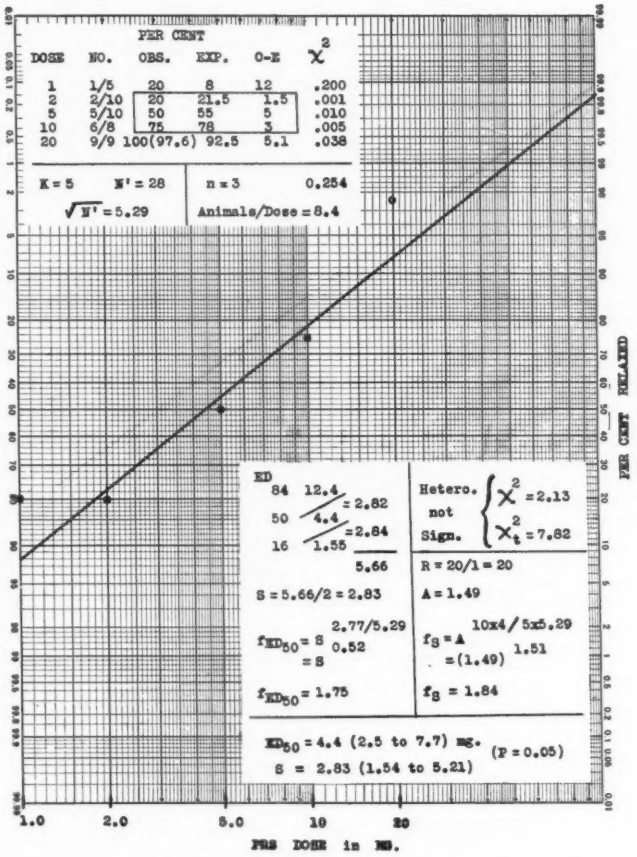

FIG. 4. Dose-per cent effect line for a single dose of an extract of pregnant rabbit serum (PRS). The per cent effect for the 20 mg. group is based on a recalculated value. For the details of this procedure, see Litchfield and Wilcoxon (1949).

bones (Table 2). In addition, the average response for each group rises with increasing dosage to a maximum of 1.15 to 1.17 mm. for the single dose, and to a maximum of 1.64 to 1.70 mm. for the double dose. This allows for two methods of assay: a qualitative method based upon the per cent of animals responding and a quantitative method relying upon the amount of separation which occurs in the various groups.

The per cent-response for a single dose of PRS was examined by the method of Litchfield and Wilcoxon (1949) (Fig. 4). By the test of Chi square, the line was found to be a good fit; the data were not significantly heterogeneous. The 50 per cent effective dose was 4.4 mg. and the limits

of confidence for this dose were +3.3 and −1.9 mg. The slope of the line was sufficiently steep and covered a sufficient range to be comparable to that of many other biological assay methods including those considered by Litchfield and Wilcoxon as examples. These facts did not hold true for the reaction to the second dose of PRS since most of the animals respond to the combined treatment. A statistical analysis for per cent response to 2 doses of PRS would therefore not be profitable.

The line relating mm. response to log-dose for a single dose of PRS (Fig. 5) has been drawn between the dose levels of 2.0 and 20 mg. according to the procedures described by Pugsley (1946), Sayers *et al.* (1948)

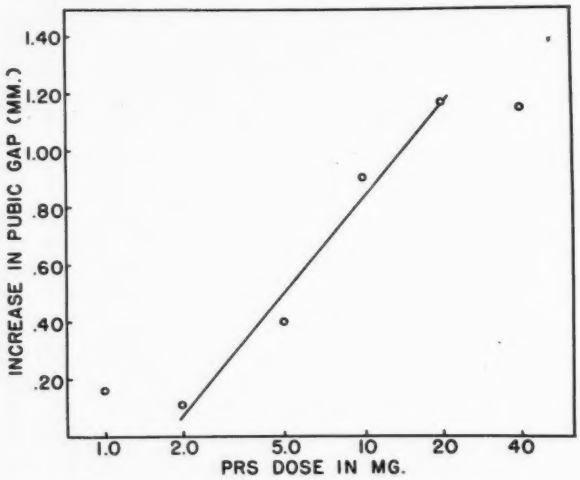

FIG. 5. Single dose assay of an extract of pregnant rabbit serum (PRS).

and Emmens (1950). The slope of the line (b) is 1.09 and the standard deviation about the line is 0.10 mm. The index of precision (s/b or λ) is then found to be 0.092 and the 5 per cent confidence limits are ± 0.20 mm.

No attempt has been made to examine the line based upon two doses of PRS because an insufficient number of points fall on the rectilinear portion of the curve. However, there is a favorable increase in slope compared to the curve for a single dose. The variation also seems to be increased and the range suitable for assay seems to be unchanged.

In the past, the response of the mouse to extracts of pregnant sow ovaries (PSO) has sometimes differed from the response to pregnant rabbit serum (PRS). It seemed desirable to study the effect of PSO in an assay range comparable to that of PRS. A single dose assay was performed

for a crude 5% saline extract of pregnant sow ovaries. Intact mice were used for this assay of PSO, and the dosages were chosen with the intention of obtaining more points on the rectilinear portion of the assay curve. The data are summarized in Table 3 and in Figure 6.

TABLE 3. ASSAY OF PREGNANT SOW OVARY EXTRACT (PSO)

Number of mice	Total dosage (mg.)	Per cent relaxed	Response ± S.E. (mm.)	Standard deviation
4	1.25	50	0.30 ± 0.25	0.67
10	2.50	60	0.49 ± 0.13	0.41
9	5.00	78	0.63 ± 0.14	0.43
9	10.00	78	0.79 ± 0.20	0.59
7	15.00	86	1.04 ± 0.16	0.32

$b = 0.63$
$s = 0.062$*
$\lambda = 0.10$
* Defined in Table 2.

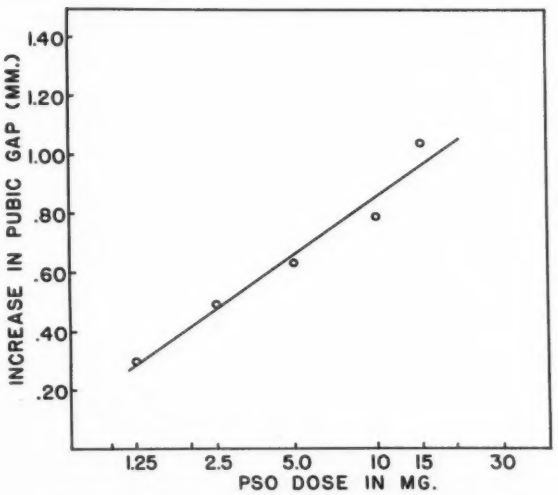

FIG. 6. Single dose assay of an extract of pregnant sow ovaries (PSO).

A line based upon per cent response again proved to be a good fit to the experimental data but, in this case, the line had a very low slope and wide confidence limits, both of which were unfavorable for assay purposes. The quantitative responses are better in this respect. The doses plotted in Figure 6 cover somewhat more than a ten fold range. The slope (b) of this line is 0.63, the standard deviation is 0.062 mm. The index of precision (s/b or λ) is 0.10 and the 5 per cent confidence limits are ± 0.12 mm.

A Chi-square comparison of the two quantitative assay curves for PRS and PSO (Figs. 5 and 6) indicates that there is no significant difference

between the slopes, possibly because of the large variation found among the animals in the various experimental groups.

DISCUSSION

At the start of this investigation, we examined the conditions which Hall (1948) considered satisfactory for the roentgenographic measurement of pubic separation in the mouse. The present results essentially confirm Hall's findings that 16 to 24 hours are required to obtain the maximum response increase in the pubic gap of the mouse following a single injection of the relaxative hormone. While the entire duration of the separated state was not examined in this study, closure of the pubic symphysis did not occur during 36 hours. This is also in agreement with Hall (1948) who has reported that the symphysis starts to close 48 hours after a single injection of pregnant rabbit serum.

It should be noted that from the technical point of view, this assay procedure is not a complex one. Since the animals need not be castrated, it entails only a series of injections and two X-ray photographs of the pelvis of each animal. Moreover, the X-ray technique and the projection method for measurement has proven to be extremely accurate. Repeated X-ray photographs of the same mouse and repeated measurements of individual photographs yielded results with small variation.

The statistical analysis of the assay curves demonstrate that the relation between log-dose and mm. response is linear over a sufficiently wide range to permit a satisfactory assay of most materials. While variation is larger than is desirable, it is certainly not outside the limits of practicality. The values for λ, the coefficient of variability, are about 10% which is common for bioassays.

There is general agreement that the desirable unit for a biological preparation of unknown purity is based on comparison with a standard. Since no standard ovarian powder exists, we have temporarily used a "laboratory unit" for purposes of chemical fractionation of the active principle and for the measurement of the amounts of the hormone in various biological extracts. After a preliminary test, the "unknown preparation" is assayed in three groups of six to eight mice at dosage levels sufficient to produce increments in the pubic gap which fall between 0.3 and 1.1 mm. A straight line is drawn through the points plotted for log-dose vs. mm. response and a mouse unit is taken as that amount of the extract which produces an average increment of 0.65 mm. In practice, it is possible to use the mice again by giving each of the three groups a second dose of another extract immediately after the response to the first dose has been recorded. The response to this second dose is only used as a preliminary test in preparation for the next complete assay. When this procedure

is followed, the assay of each new "unknown" requires only 18 to 24 new mice.

The problem of reproducibility of results for this type of assay unit remains a serious one. To test for the variation of response, the same preparation was assayed at intervals of several weeks, each time in a single group of five randomly chosen intact mice. The results were 0.78 ± 0.15 mm., 0.88 ± 0.22 mm., and 0.88 ± 0.23 mm. (Mean $\pm$ S.E.). It is hoped that a standard ovarian powder will soon be established so that all units can be defined in terms of the standard preparation.

ADDENDUM

Recently Dorfman *et al.* (1953) reported that relaxin can be assayed by its effect on the mouse pubic symphysis, but stated that single daily doses of relaxin were unable to maintain pubic separation even when repeated over a number of days. We find that single doses of PRS or PSO are very effective in producing and maintaining large pubic separations. For instance, these investigators obtained increments of the order of 0.10 to 0.15 mm. with two doses of relaxin in 24 hours, whereas single doses of our crude extracts produce increments of 1.00 to 1.15 mm. in 24 hours. Even for a four dose, 48 hour assay response, Dorfman and his co-workers failed to explore more than the lower fourth of the potential assay curve. A previous publication (Kliman and Salhanick, 1951) and work still in progress indicate that relaxin of high potency with respect to pubic relaxation in guinea pigs is of much lower potency than crude extracts when tested in the mouse.

SUMMARY

Mature, virgin female mice were used for the bioassay of a relaxative hormone present in crude extracts of pregnant rabbit serum (PRS) and pregnant sow ovaries (PSO). Following seven days pretreatment with 1 μg. estradiol 17β daily, a single dose of either extract produced significant separation of the pubic symphysis. The response was measured by means of X-ray photographs of the pelvis. A twofold increase in sensitivity to the hormone was observed when the estradiol priming period was lengthened to 23 days. Maximal separation of the pubic symphysis of the mouse occurred within 16 to 24 hours after a single injection of ovarian extract. Intact or castrated mice respond equally well.

A statistical analysis of the assay data indicated that reliable quantitative assays were obtained. Lacking a standard powder, a provisional unit of activity (mouse unit) was defined as follows:

That amount of relaxative hormone which will produce a mean increment of 0.65 mm. in the separation of the pubic symphysis, when given

as a single injection to a group of 6 to 8 mice pretreated with 1 μg. of estradiol for 7 days.

Acknowledgements

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A COMPARISON OF THE RESPONSE OF THE PUBIC SYMPHYSIS OF THE MOUSE AND GUINEA PIG TO EXTRACTS OF PREGNANT SOW OVARIES[1]

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THE response of the pubic symphysis of the estrogen-primed mouse to relaxin-containing extracts of pregnant rabbit serum (PRS) and of pregnant sow ovaries (PSO) has been reported previously (Kliman, Salhanick and Zarrow, 1953). Significant symphyseal separations were obtained with both types of extracts, although some differences were noted in the slopes of the dose-response curves of preparations from each of these two sources. However, roentgenographic measurement of the average increments in pubic separation produced by single doses of such extracts gave reliable quantitative determinations of activity. The effectiveness of crude extracts was confirmed by their ability to produce significant relaxation in all treated mice, and to reproduce the rate of separation which normally occurs during pregnancy.

Subsequently, studies were made of the action, on the pubic symphysis of the mouse, of relaxin preparations of different specific activities. It has been found that some preparations which are very potent in the guinea pig are only slightly active in effecting pubic separation in the mouse. It has also been possible to partially purify material in which the ratio of mouse to guinea pig activity is substantially higher than that of the original crude extracts of sow ovaries. Finally, this paper reports experimental evidence concerning the nature of the relaxative agent(s) present in extracts of the ovaries of pregnant sows.

MATERIALS AND METHODS[4]

Ovarian extracts: Preparations PSO and K-1 were prepared from ovarian homogenates by extraction with 5% NaCl solution (4/L./kg.). The extract, after centrifugation to

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[4] The authors are indebted to Dr. Thomas C. Smith for preparation R-S1 and to Dr. Robert L. Kroc of Chilcott Laboratories, for his assay of preparation X-4.

remove insoluble residue, was dialyzed against running tap water and then lyophilized. R-S1 was prepared according to the method of Frieden and Hisaw (1950), and preparation 52-2 was the more highly purified material discussed in that publication. The preparation of X-4 is described below.

Assay procedures: Activity in the guinea pig was determined by the method described by Frieden and Hisaw (1950). Each preparation was tested at a minimum of 3 dose levels; the results are expressed in guinea pig units (GPU) per milligram. Activity in the mouse was measured by the procedure of Kliman, Salhanick and Zarrow (1953). Both the proportion of mice responding and the average increments in pubic separation were recorded; the calculation of specific activities (mouse units (MU) per mg.) was based upon the latter data.

RESULTS

Extracts of ovaries of pregnant sows, prepared by the various procedures described above, were assayed in both mice and guinea pigs (Table 1 and Fig. 1). The crude ovarian extracts, preparations K-1 and PSO gave proportionate results in both species. Parallel dose-response curves were obtained for these two preparations in mouse assays and the ratio of

TABLE 1. THE RELATIVE ACTIVITIES OF VARIOUS PREPARATIONS IN THE MOUSE AND GUINEA PIG

Preparation	GPU/mg.	MU/mg.	GPU/MU
X-4	0.07	0.20	0.35
K-1*	1	0.10	10
PSO*	2	0.20	10
R-S1	50	<0.25[1]	>200
52-2 (one dose)	140	—[2]	—
52-2 (three daily doses)	140	<0.13[2]	>1100

* Initial saline extract of pregnant sow ovaries.
[1] Some activity demonstrable at a dosage level of 4 mg. per mouse. See text.
[2] No significant activity demonstrable at levels of 0.2 and 0.6 mg. respectively. The activity ratio was obtained by extrapolation of the few minimal responses which 52-2 produced in mice at the highest dose tested.

guinea pig to mouse activity was approximately the same for both preparations (10 GPU = 1 MU). This ratio has also been reported for extracts of blood serum of pregnant rabbits (Kliman, Salhanick and Zarrow, 1953).

Preparations R-S1 and 52-2 contained little mouse activity despite the fact that both were very effective in the guinea pig (50 and 140 GPU per mg. respectively). The pubic symphyses of the mice used for these assays never attained the degree of separation characteristically obtained with unfractionated extracts. Furthermore, the proportion of animals which responded with significant separations was rarely greater than 50% and the dose-response curves were characterized by low slopes (b) and high variability (λ) (Table 2 and Fig. 1). However, it should be emphasized that in each group of mice treated with these preparations, a few mice always attained highly significant separations of about 0.8–1.0 mm. Under

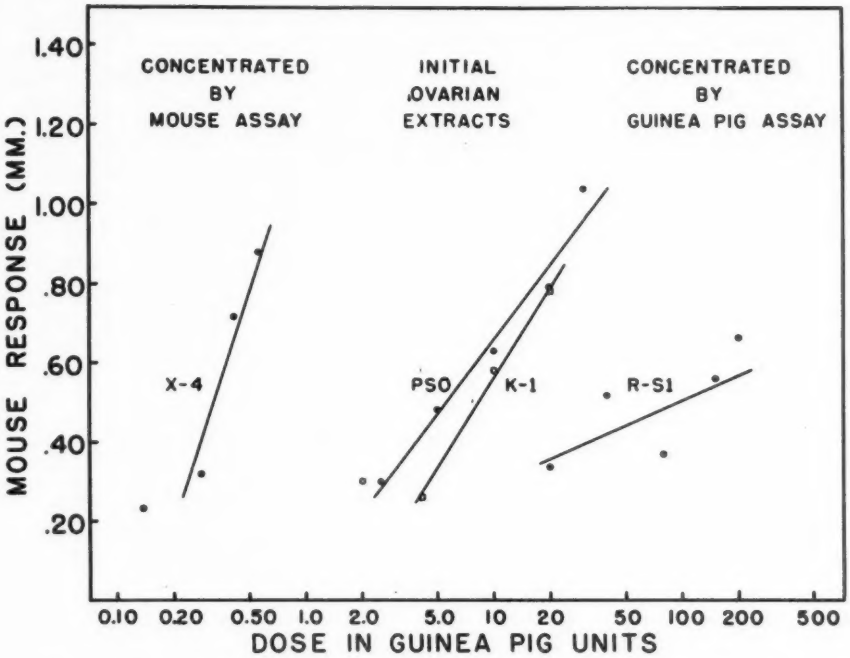

FIG. 1. Assays, in the mouse, of relaxin-containing ovarian extracts. Note the lack of correlation between activity in the guinea pig (GPU) and effect in the mouse (mm. increment in pubic separation).

similar conditions, the maximal separations obtained with crude extracts range from 1.2 to 1.5 mm.

When a crude saline extract of sow ovaries was dialyzed against an equal volume of cold 95% ethanol, the resulting precipitate contained substantially all the mouse activity of the original extract. Almost all of the guinea pig activity was found in the supernatant fraction. The precipitate was again extracted with 5% NaCl and was then reprecipitated from 50% ethanol. The precipitate, preparation X-4, was lyophilized.

TABLE 2. ASSAYS OF RELAXIN CONTAINING EXTRACTS OF PREGNANT SOW OVARIES IN THE MOUSE

Preparation*	No. of mice	Range assayed (GPU)	Slope (b)	S.E. of Estimate (s)	Coefficient of variability (λ)
X-4	24	0.14– 0.56	1.53	0.096	0.063
K-1	20	2 – 20	0.74	0.025	0.034
PSO	39	2.5 – 30	0.63	0.062	0.100
R-S1	57	20 –200	0.21	0.105	0.495

* Preparation 52-2 was essentially inactive in the mouse in the highest dose tested and is therefore not included in this table.

On assay in the mouse it displayed a dose-response curve which was significantly steeper than that found for the initial crude extract. Two groups of workers independently assayed X-4 in the guinea pig and found that it contained only slight activity (Table 1). As compared with unfractionated extracts, this sample represents a twofold concentration of mouse activity and a concomitant 15 to 30-fold decrease in guinea pig activity.

Relaxin preparations have been shown to retain their effectiveness in the guinea pig following exposure to 100° C. for 15 minutes (Frieden, 1951b), but are unstable in the presence of a variety of reducing agents, including cysteine (Frieden, 1951a). Studies of the effects of heat and of reduction, with 0.04 M cysteine at room temperature and pH 7.8 for 16 hrs., showed that the mouse relaxative agent shared these properties (Table 3).

TABLE 3. EFFECTS OF HEAT AND OF CYSTEINE TREATMENT UPON THE ACTIVITY OF PREPARATION X-4 IN THE MOUSE

Group	No. of mice	Dose	Response in mm. (Mean ± S.E.)	M.U./10 mg. aliquot
I	8	10 mg. X-4	0.94 ± 0.26	2
II	7	10 mg. X-4 (cysteine-treated)*	0.01 ± 0.026	0
III	7	Filtrate from 10 mg. X-4, after heating†	0.37 ± 0.08	0.7
IV	7	Precipitate from 20 mg. X-4, after heating	0.81 ± 0.07	0.75

Application of Student's "*t*" test to groups I and II revealed that the difference between these groups was highly significant ($t = 3.32$ and $P = < 0.01$ for 13 degrees of freedom).

* 0.04 M cysteine, pH 7.8, 16 hr. at 26°.

† One hundred degrees C., 15 min.

A striking difference between guinea pig-active and mouse-active extracts was demonstrated by treatment of both types of extracts with urea solution. Following exposure to 6 M urea for 16 hours, at room temperature and neutral pH, each preparation was dialyzed against running tap water for 24 hours and was then assayed. Ten mg. of preparation X-4, untreated, produced a response of 0.84 ± 0.18 mm. (Mean ± S.E.) in a group of eight mice. Twice this amount of urea-treated X-4 produced a response of 0.08 ± 0.025 mm. in a like number of animals. In sharp contrast, no detectable decreases in guinea pig activity could be observed following treatment of either crude or partially purified relaxin extracts with urea.

In an attempt to evaluate the significance of pharmacological factors in the response of the mouse to purified relaxin preparations, studies were made of the effects of these preparations when administered in such a way as to reduce the rate of absorption. Admixture of relaxin with gelatin or alum (method of Hayashida and Li, 1952), local infiltration into the symphyseal region, and intramuscular injection all failed to produce a signifi-

cant increase in response compared to the effects when the same preparation was given subcutaneously. On the other hand, a significant decrease in the effectiveness of preparation X-4 upon intraperitoneal injection was recorded in two separate experiments; similar results were obtained in a third experiment with a massive (200 GPU) dose of preparation R-S1 (Table 4). No such reduction in activity is observable when relaxin is injected intraperitoneally in the guinea pig (Zarrow and Money, 1948). It seems likely that the differences noted reflect the fact that the mouse requires 16–24 hours for maximal relaxation (Kliman, Salhanick and Zarrow, 1953) whereas the guinea pig responds within 4–6 hours (Abramowitz, *et al.*, 1944).

TABLE 4. EFFECT OF THE ROUTE OF INJECTION ON THE ACTIVITY OF EXTRACTS R-S1 AND X-4 IN THE MOUSE

No. mice per group	Dose assayed	Route	Response in mm. (mean ±S.E.)	t	P (less than)
6	10 mg. X-4	Subc.[1]	1.10 ±0.23	3.10	0.01
12	10 mg. X-4	I.P.[2]	0.48 ±0.08		
11	10 mg. X-4	Subc.	0.83 ±0.11	2.31	0.05
10	10 mg. X-4	I.P.	0.46 ±0.11		
7	4 mg. (200 GPU) R-S1	Subc.	0.67 ±0.19	2.40	0.05
8	4 mg. (200 GPU) R-S1	I.P.	0.19 ±0.08		

[1] Subcutaneous injection.
[2] Intraperitoneal injection.

DISCUSSION

The experiments reported above indicate that the pubic symphysis of the mouse and the guinea pig relax in response to two different fractions obtainable from the ovaries of pregnant sows. That these fractions may not be completely unrelated is suggested (1) by their occurrence in approximately the same proportions in unfractionated ovarian extracts as well as in pregnant rabbit serum, (2) by certain similarities in chemical properties, and (3) by the fact that significant pubic separations almost invariably occur in some of the mice treated with purified relaxin preparations. The latter phenomenon cannot be explained on the basis of contamination of purified extracts with small amounts of mouse-active material, since it would be expected that only the intercepts, and not the slopes, of the dose-response curves would be different.

That relaxin exists in complex form in tissue extracts has been suggested previously (Frieden and Hisaw, 1950). Although little direct evidence can be adduced for the view that the substance which is effective in the mouse is an association complex of relaxin with other tissue proteins, this hypothesis merits consideration, since it would account for the differences

in solubility properties which have been observed, as well as for some similarities in chemical properties.

The problem of the identity of the human relaxative hormone is an interesting one. When the guinea pig assay is used, relaxin can be detected in human pregnancy sera (Pomerenke, 1934; Abramson *et al.*, 1937; Zarrow *et al.*, 1953). Current experiments using mouse assays have shown no activity in extracts of sera taken at various stages of human pregnancy (Salhanick *et al.*, unpublished observations), although the serum of pregnant rabbits is active in both the mouse and the guinea pig. The significance of these differences in activities of sera and of extracts of ovaries of pregnant sows with respect to the problem of the chemical nature of the relaxative hormone is not clear. It is possible that at least two chemically distinct but closely related substances affecting pelvic relaxation exist and that there may be a species difference as to whether one or both are formed by the tissues of the reproductive tract.

SUMMARY

A series of relaxin-containing extracts from ovaries of pregnant sows were compared for their effectiveness in producing pelvic relaxation in mice and guinea pigs. Material concentrated on the basis of the guinea pig assay was only slightly effective in the mouse as shown by roentgenographic measurement of pubic separation. Conversely, material concentrated in respect to assay in the mouse contained less than 5% of the original guinea pig activity. When partially purified relaxin (guinea pig assay) was tested in the mouse, an atypical dose-response curve of low slope and high variability was observed.

The use of different routes of administration, and the addition of adjuvants (gelatin; alum) to preparations of high specific guinea pig activity failed to restore activity for the mouse.

The two types of relaxative hormone extracts were similar in resistance to heating and with respect to inactivation on exposure to cysteine. However, six molar urea solution inactivated a mouse-active extract but did not diminish guinea pig activity. It is suggested that the mouse-active fraction may represent the binding of relaxin to a less soluble, more slowly mobilized carrier molecule.

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THE INFLUENCE OF THE ADRENAL CORTEX AND COLD STRESS ON FASTING KETOSIS IN THE RAT

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KETOSIS has been considered to be a part of the "Alarm Reaction" and as such conceivably mediated by or dependent upon adrenal cortical activity (Selye, 1950; Sargent and Consolazio, 1951), although there are few concrete data on which to base such a conclusion. Admittedly ketosis may occur following a variety of stressful stimuli such as muscular effort (Gemmill, 1934; Errebo-Knudsen, 1946; Drury *et al.*, 1941a; Neufeld and Ross, 1943; Taylor *et al.*, 1945), reduced atmospheric pressure (Evans, 1936), surgical trauma (Roscher, 1933; Sachar *et al.*, 1950; Elman and Weichselbaum, 1951), x-irradiation (Kikuchi, 1931), artificial fever (Somogyi and Kirstein, 1940) and even in response to stressful life situations (Hinkle *et al.*, 1950), but little is known concerning the metabolic mechanisms involved in this type of ketosis and even less about possible hormonal influences. The adrenal cortex is activated under these same circumstances, but there is no adequate experimental basis for attributing the metabolic response of ketosis to either a direct or even a permissive action of adrenal steroids. This is particularly true since even now there is still no agreement concerning the precise role of the adrenal cortex in ketosis under any circumstance.

Adrenalectomy generally reduces but does not necessarily always abolish the ketosis of fasting (MacKay and Wick, 1937), phloridzination (Evans, 1936; Segaloff and Many, 1952), reduced atmospheric pressure (Evans, 1936), as well as that after the administration of anterior pituitary extract (Fry, 1936, 1937; MacKay and Barnes, 1937; Neufeld and Collip, 1938; Mirsky, 1938; Shipley, 1940). Certain inconsistencies in reports on the effects of adrenalectomy on ketosis under various conditions may be explicable on the basis of the recent finding that peripheral circulatory

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failure results in a striking inhibition of ketosis (Engel and Hewson, 1953). It is well known that the adrenalectomized animal is frequently in incipient, if not overt, peripheral circulatory failure, particularly when subjected to stress.

The depressed ability of the liver to make ketone bodies *in vitro* after adrenalectomy or hypophysectomy has been restored by treatment with small amounts of cortisone (Lipsett and Moore, 1951, 1952; Tepperman and Tepperman, 1951). Segaloff and Many (1952) report that lipoadrenal extract, hydrocortisone and corticosterone will not only restore but will increase above normal the ketonuria of phloridzinized-adrenalectomized rats. Bennett and collaborators have reported a prompt ketonemia in dogs (1948a) and rats (1948b) after a single injection of ACTH, while Thorn *et al.* (1948) described a small increase in blood ketone levels of a fasting Addisonian patient receiving cortisone. The rises in blood ketones in the rats were clear cut, but those in the dog and man were very small and of questionable significance. Bondy and Wilhelmi (1950) could not confirm Bennett's results with ACTH, although their rats exhibited significant ketone elevations after growth hormone. Shipley and Fry (1941) could detect no increase in ketonemia in rats treated with cortisone, corticosterone or desoxycorticosterone, while recently Kinsell *et al.* (1951) not only found no increase in fasting ketosis, but actually a decrease in blood ketone levels of humans fasted 72 hours and given ACTH or cortisone. Comparable results have been observed in this laboratory in rats and man (Engel and Engel). The contradictory results in the literature just described are not clarified by the recent reports of Ingle *et al.* (1951a, 1951b) that rats force-fed high fat diets developed transient ketosis when receiving large amounts of ACTH or cortisone.

The present studies were designed to elucidate some of the factors involved in the ketosis of stress and its relation to the adrenal cortex. For this purpose, cold exposure was chosen as the stressor and the effects of ACTH, cortisone and hydrocortisone tested. It was found that cold exposure resulted in a marked increase in fasting ketosis, while cortisone and hydrocortisone treatment suppressed fasting ketosis, both at normal and low environmental temperatures.

METHODS

Male albino rats of the Vanderbilt strain and similar weight were used. Prior to the fasting period, they were maintained on a diet of commercial chow, supplemented with liver pudding and lettuce twice weekly (Engel, Schiller, and Pentz, 1949).

Animals receiving cortisone acetate and hydrocortisone acetate were given a subcutaneous injection of a saline suspension of the steroids 48 and 24 hours prior to the fasting period, and at daily intervals thereafter. In the experimental groups designated

as chronic cortisone-treated, injections of cortisone were administered daily for seven days prior to the onset of fasting, and each day thereafter during the fast. ACTH (Armour Lot #J17409R) in physiologic saline solution was administered in four 1 mgm. injections at approximately 5-hour intervals daily, with a maximum overnight lapse between injections of 10 hours. No pretreatment with ACTH was given, the first injection being made at the onset of fasting.

Tail blood for ketone and glucose determinations was obtained without anesthesia, before the onset of fasting and at 24-hour intervals thereafter. Bleeding was performed at the same approximate time each day, to obviate the possibility of diurnal variations and to reduce differences in the time period following the last injection of hormone in those animals receiving ACTH. Blood sugar and ketone levels were determined on samples of barium hydroxide-zinc sulfate protein-free filtrates. Total blood ketones, as acetone, were determined by a modification of the method of Greenberg and Lester (1944, 1948), and blood sugar by the method of Somogyi (1945).

Following the initial bleeding, all animals were fasted with ad libitum water intake in individual cages. Those designated as cold stressed were placed in a room maintained at 4° C. The remainder were kept in an air-conditioned room at a temperature of 25–27° C.

Animals at room temperature were sacrificed at the end of five to seven days of fasting and the adrenals removed and weighed on a Roller-Smith torsion balance. Adrenal weights are expressed as mg. 100 gm. of body weight before fasting. Due to the reduced survival of cold-stressed animals, adrenal weights could only be determined at the time of spontaneous demise of these animals. For this reason, mean survival in each group is indicated in Tables 3 and 4.

RESULTS

The effects of fasting and hormone treatment on blood sugar and ketone levels in unstressed animals is shown in Table 1 and in cold stressed rats in Table 2. During an uncomplicated fast, blood ketone levels rose to high levels during the first two days, and then slowly declined, although on the fourth day they were still significantly elevated. Blood sugar levels varied inversely with the ketone levels, being lowest on days one and two and highest on days three and four. On treatment with either 5 or 10 mg. of cortisone acetate or 5 mg. of hydrocortisone acetate daily, fasting ketosis was considerably modified. Not only was there no appreciable rise in blood ketones during fasting but in almost all cases blood levels in the hormone treated rats were lower during fasting than in the corresponding controls. Blood sugar levels were significantly elevated during steroid treatment and again exhibited an inverse relationship to the ketonemia. In these experiments, the steroid treatment was begun 48 hours before the fast to insure absorption of an adequate amount of hormone by the time the fast began.

In contrast to the clear-cut effects of cortisone and hydrocortisone in suppressing ketosis was the relative ineffectiveness of ACTH when administered in a dose of 1 mgm four times a day. Although blood sugar levels on days 0, 1 and 2 were significantly higher in the ACTH group than in the

TABLE 1. EFFECTS OF ACTH, CORTISONE ACETATE, HYDROCORTISONE ACETATE AND COLD STRESS ON FASTING KETOSIS

	Fasting days				
	0	1	2	3	4
	Blood ketones mg./100 ml.				
Control	1.07 ± 0.07 (8)	4.12 ± 0.28 (9)	4.21 ± 0.46 (9)	2.75 ± 0.48 (8)	3.70 ± 0.76 (8)
Cold stressed	1.01 ± 0.11 (10)	*7.27 ± 0.48* (9)	*8.03 ± 0.60* (10)	*4.98 ± 0.40* (8)	2.23 ± 0.25 (7)
Cortisone 5 mg.	1.37 ± 0.31 (6)	*1.08 ± 0.15* (6)	*1.62 ± 0.72* (6)	1.19 ± 0.17 (6)†	0.92 ± 0.22 (6)
Cortisone 10 mg.	1.38 ± 0.32 (9)	*1.56 ± 0.13* (4)	2.22 ± 0.24 (4)‡	1.82 ± 0.19 (4)	1.63 ± 0.35 (4)
Compound F 5 mg.	1.68 ± 0.18 (10)	*1.97 ± 0.19* (9)	*1.74 ± 0.17* (6)	2.12 (3)	
ACTH 1 mg. QID	1.10 ± 0.08 (8)	3.97 ± 0.22 (8)	3.84 ± 0.41 (8)	3.16 ± 0.35 (8)	4.06 ± 0.49 (8)
Chronic cortisone 10 mg.	1.03 ± 0.10 (4)	*2.54 ± 0.14* (4)	*1.52 ± 0.10* (4)	1.62 ± 0.14 (4)	1.34 ± 0.13 (4)‡
	Blood glucose mg./100 ml.				
Control	93 ± 4.8 (6) P < 0.01	68 ± 1.3 (6)	74 ± 4.2 (6)	80 ± 3.3 (5)	88 ± 6.2 (4)
Cold stressed	101 ± 3.2 (7) P < 0.01	71 ± 1.9 (6)	85 ± 3.5 (7)	*107.6.2* (5)	118 ± 15.4 (5)
Cortisone 5 mg.	*118 ± 2.9* (6) P < 0.2	*109 ± 6.4* (6)	*112 ± 5.8* (6)	*116 ± 7.3* (6)	*115 ± 3.8* (6)
Cortisone 10 mg.	*124 ± 4.0* (9) P < 0.05	*109 ± 3.0* (4)	*107 ± 1.8* (4)	*118 ± 2.3* (4)	*114 ± 3.1* (4)
Compound F 5 mg.	*123 ± 3.0* (10) P < 0.1	*115 ± 4.8* (9)	91.5 (2)		
ACTH 1 mg. QID	*111 ± 2.6* (8) P < 0.01	*89 ± 3.3* (8)	*91 ± 3.4* (8)	90 ± 2.4 (8)‡	84 ± 3.6 (8)
Chronic cortisone 10 mg.	*143 ± 7.8* (4) NS	*129 ± 5.6* (4)	*121 ± 9.1* (4)	102 ± 3.5 (4)	113 ± 4.5 (4)

Results expressed as mean ± standard error. Number in parentheses refers to number of observations.

Italicized values indicate $P < 0.01$ as compared with control group.

† $P < 0.02$ as compared with control group.

‡ $P < 0.05$ as compared with control group.

P significance between initial and 24-hour blood glucose in same experimental group.

controls, no difference between the ketone values was discernible.

Since Ingle *et al.* (1951a) found that sporadic ketonuria occurred in rats force-fed a high fat diet only after 7 to 8 days of cortisone treatment, an additional group of animals was observed in which cortisone treatment was begun seven days before the fast. Ten mgm per day were given, conforming to the dose used by Ingle. The response in both ketonemia and glycemia was entirely comparable with that previously noted with cortisone or hydrocortisone treatment.

The second row of data in Table 1 which corresponds with the first row in Table 2 portrays the effect of cold exposure on fasting ketosis. Note that ketonemia is strikingly increased during days one, two and three of the

TABLE 2. EFFECTS OF ACTH, CORTISONE ACETATE AND HYDROCORTISONE ACETATE ON FASTING KETOSIS DURING COLD EXPOSURE

	Fasting days				
	0	1	2	3	4
	Blood ketones mg./100 ml.				
A Control	1.01 ± 0.11 (10)	7.27 ± 0.48 (9)	8.03 ± 0.60 (10)	4.98 ± 0.40 (8)	2.23 ± 0.25 (7)
B Cortisone 5 mg.	1.08 ± 0.22 (7)	*3.01 ± 0.27* **(7)	*4.52 ± 0.87* (7)	*2.54 ± 0.62* **(6)	1.09 ± 0.15 (4)‡
C Cortisone 10 mg.	1.38 ± 0.32 (9)‡	*4.08 ± 0.34* **(7)	5.56 ± 0.72 **(7)‡	*2.29 ± 0.58* (6)	*0.61 ± 0.05* (4)
D Compound F 5 mg.	1.37 ± 0.08 (8)‡	*3.76 ± 0.24* **(8)	*5.02 ± 0.52* **(8)	5.51 ± 0.88 (6)	3.45 ± 0.73 (5)
E ACTH 1 mg. QID	1.26 ± 0.11 (11)	5.43 ± 0.44 (11)‡	5.63 ± 0.96 (7)‡	3.97 ± 0.89 (6)	
F Chronic cortisone 10 mg.	1.18 ± 0.17 (5)	6.20 ± 0.64 **(5)	5.47 ± 0.98 **(5)§		
	Blood glucose mg./100 ml.				
A	101 ± 3.2 (7) P < 0.01	71 ± 1.9 (6)	85 ± 3.5 (7)	107 ± 6.2 (5)	118 ± 15.4 (5)
B	114 ± 4.7 (6)§ P < 0.01*	*86 ± 2.6* (7)	92 ± 2.2 (7)	114 ± 3.4 (6)	132 ± 8.9 (4)
C	*124 ± 4.0* (9) N.S.	*118 ± 5.3* (7)	109 ± 4.4 (7)§	138 ± 9.2 (6)§	125 ± 4.3 (4)
D	*128 ± 5.9* (8) P < 0.02	105 ± 6.0 (8)‡	*102 ± 4.5* (8)	95 ± 8.7 (3)	105 ± 6.2 (4)
E	108 ± 1.8 (12) P < 0.01	78 ± 3.8 (12)	85 ± 5.7 (7)		
F	*121 ± 4.0* (5)	*119 ± 1.9* (4)	93 ± 2.2 (4)		

Italicized values indicate $P < 0.01$ as compared with control group.
‡ $P < 0.02$ as compared with control group.
§ $P < 0.05$ as compared with control group.
P Significance between initial and 24-hour blood glucose in same experimental group.
** Ketones higher in cold-stressed as compared with unstressed receiving same treatment $P < 0.01$.
* Blood glucose lower in cold-stressed as compared with unstressed receiving same treatment $P < 0.01$.

fast in the cold compared with the controls, but that the high levels decline by the fourth day. Blood sugar levels were comparable to the controls except that those on the third and fourth days were rather higher than the controls.

The effects of adrenal hormone treatment on fasting ketonemia and glycemia during cold exposure are seen in Table 2. While 5 and 10 mg. of cortisone-acetate and 5 mg. of hydrocortisone acetate all proved effective in decreasing blood ketone levels below those of the untreated cold exposed rats, ketonemia, nevertheless, generally was significantly greater than in the corresponding unstressed steroid treated groups (Table 1). The lower dose of cortisone proved less effective in maintaining the blood sugar in the cold stressed group than in the unstressed control, but with 10 mg. of

cortisone and 5 mg. of hydrocortisone blood sugar levels were quite comparable in the control and cold exposed group. It is significant that ketosis still occurred despite the elevated blood sugars. In contrast to the lack of effect of ACTH in the control, this hormone proved partially effective in suppressing ketosis in the cold-stressed group. On the other hand, pretreatment with 10 mg. of cortisone for 7 days was followed by a distinctly lowered resistance to cold exposure and no appreciable suppression of ketosis. On the second day of fasting, the blood sugar levels of the cold exposed rats were significantly lower than in the non-stressed group receiving 10 mg of cortisone chronically.

Tables 3 and 4 depict data on weight loss, survival and adrenal weights under the experimental conditions described above. During the first 3

TABLE 3. EFFECTS OF ADRENAL HORMONES ON BODY AND ADRENAL WEIGHT DURING FASTING (MEANS ± S.E.)

Experimental group	Initial body weight grams	3 day fasting weight loss	Adrenal weight mg./100 gm. initial body weight	Mean survival days
Control	275	16%	14.5 ± 0.64 (9)	7
Cold stressed	276	23%	*18.4 ± 0.88* (9)	4
Cortisone 5 mg.	258	17%	11.7 ± 1.24 (6)‡	6
Cortisone 10 mg.	241	21%	12.1 ± 0.85	5
Compound F 5 mg.	271	17%	11.8 ± 0.54 (5)§	4.5
ACTH 1 mg. QID	261	18%	*20.2 ± 0.45* (8)	5
Chronic cortisone 10 mg.	210	22%		5

Italicized values indicate $P < 0.01$ as compared with untreated control group.
‡ $P < 0.02$ as compared with untreated controls.
§ $P < 0.05$ as compared with untreated controls.

days of fasting at room temperature, weight loss was not notably different in any of the groups except in those receiving 10 mg. of cortisone acutely or chronically where it was slightly greater. Cold exposure did increase weight loss and in the animals receiving 10 mg. of cortisone or ACTH this weight loss was greater. The control animals were all sacrificed on the seventh day, while all other groups exhibited a mean survival of less than this, with the cold-exposed hormone treated animals showing poorest survival.

The changes in adrenal weight were of considerable interest. The adrenal glands of the cold exposed animals showed significant hypertrophy by the fourth day compared with the size of glands of normal animals fasted 7 days. As might be anticipated, the adrenal glands of the control hormone treated animals were smaller than the untreated controls, but this is of

TABLE 4. EFFECTS OF ADRENAL HORMONES ON BODY AND ADRENAL WEIGHT DURING FASTING AND COLD EXPOSURE

Experimental group	Initial body weight grams	3 day fasting weight loss	Adrenal weight mg./100 gm. initial body weight	Mean survival days
Control	276	23%	18.4 ± 0.88 (9)	4
Cortisone 5 mg.	258	24%	16.8 ± 0.83 (7)**	3.5
Cortisone 10 mg.	250	28%	14.9 ± 1.41 (5)§	3
Compound F 5 mg.	266	24%	16.6 ± 1.22 (8)*	5
ACTH 1 mg. QID	263	28%	*22.9 ± 0.72* (8)**	3.5
Chronic cortisone 10 mg.	208		*11.1 ± 0.73* (5)	2.5

Italicized values indicate $P < 0.01$ as compared with untreated control group.
§ $P < 0.05$ as compared with untreated controls.
** $P < 0.01$ as compared with unstressed groups receiving same treatment.
* $P < 0.02$ as compared with unstressed groups receiving same treatment.

doubtful significance since in general the former were measured after a shorter period of fasting than the controls. The adrenal hypertrophy after ACTH treatment on the other hand is highly significant, particularly in light of the shorter fast. Ten mg. of cortisone significantly decreased adrenal hypertrophy during cold exposure. On the other hand, the smaller doses of cortisone or hydrocortisone were ineffectual in this regard. However, in no case except possibly in the group pretreated with cortisone for 7 days was adrenal activation by cold completely suppressed by hormone treatment. Unfortunately, adrenal weights were not measured in the control group pretreated with cortisone, and hence one cannot be certain that suppression of adrenal hypertrophy actually occurred in the corresponding cold exposed group. The adrenals of animals receiving ACTH during cold exposure were significantly larger than those of the control ACTH group. Pinto (1945) has reported a similar synergism in the adrenal response to estrogens and ACTH in hypophysectomized animals.

The data so far indicated that adrenal steroid treatment suppressed both fasting and cold stress ketosis. No particular correlation between evidences of adrenal activation and the response of ketosis could be demonstrated. Animals with adrenal hypertrophy due to cold exposure exhibited marked ketosis while other animals with even greater adrenal hypertrophy as a consequence of ACTH stimulation showed a lesser degree of ketonemia. Since acclimatization to cold by chronic exposure to low temperature is associated with considerable adrenal hypertrophy, the effect of this procedure on the response to a subsequent fast was studied. For this purpose, 12 animals were placed in the cold room for one month, with free access to

food and water and then fasted. As seen in Table 5, survival was poor, only 3 animals surviving the fast and these only for 2 days. However, the results are clearcut in that no significant ketosis occurred despite a greater fall in blood sugar on fasting and marked adrenal hypertrophy compared with unacclimatized controls.

TABLE 5. BLOOD KETONE AND GLUCOSE LEVELS IN COLD ACCLIMATIZED FASTING RATS (MEAN ±S.E.)

Initial body weight grams	Blood ketones mg./100 ml.			Blood glucose mg./100 ml.			Adrenal weight mg./100 gm. initial body weight	Mean survival days
	Fasting days			Fasting days				
	0	1	2	0	0	2		
* 267	1.59±0.09 (3)†	*2.44±0.01* (3)	*1.24±0.13* (3)	91±11.9 (3) N.S.	66±5.0 (3)	*46±8.0* (3)	*28.6±0.23* (3)§	2

Italicized values indicate $P<0.01$ as compared with untreated cold-stressed group (Tables 2 and 4).
† Higher than untreated group not exposed to cold: $P<0.01$ (Table 1).
§ Larger than ACTH-treated cold-stressed group: $P<0.01$ (Table 4).
* Animals selected for cold acclimatisation were larger (270–310 gm.) than those of other experimental groups; the above value is mean weight at the end of one month's exposure to cold. Fasting weight loss was unreliable due to irregular survival, and is not quoted.

Discussion: The suppression of fasting ketosis by cortisone and hydrocortisone in these experiments is definite and in good agreement with the results of Kinsell *et al.* (1951) in man. Blood sugar levels were consistently elevated after steroid therapy. It may be presumed that liver glycogen levels were likewise elevated except possibly in the group that received 10 mg. of cortisone acetate for seven days prior to fasting, since it has been shown by Winter *et al.* (1950) that liver glycogen levels are normal after eight to ten days of treatment with cortisone. The finding that ketosis is inhibited by cortisone and hydrocortisone is also in agreement with the results of Segaloff and Many (1952) in their study on ketonuria in phloridzinized adrenalectomized rats treated with various adrenal steroids. These investigators could elicit no increase in ketone excretion with cortisone and hydrocortisone but with certain other steroids such as compound A, acetate and corticosterone, ketone excretion was restored to normal.

In the present experiment, ACTH had no effect on fasting ketosis, although the change in blood sugar and adrenal weights of the treated animals attests to the activity of the hormone. This result is in agreement with Bondy and Wilhelmi's findings (1949) but not with Kinsell *et al.* (1951) who found a suppression of fasting ketosis by this agent in man or with the results of Bennett *et al.* (1948) and of Segaloff and Many (1952). The former reported ketonemia after ACTH in rats while the latter described increased ketonuria during ACTH treatment in phlorizinized rats. However, in this laboratory data have been accumulated on the effects of ACTH on ketonemia in rats which suggest that some preparations of ACTH may be contaminated with a ketogenic factor, presumably growth hormone (Engel and Engel). This may account for the discrepancies just

noted. In this regard, it is interesting that Segaloff and Many's rats receiving ACTH exhibited a decreased nitrogen excretion, concomitant with ketonuria and glycosuria, a response more to be expected from growth hormone than ACTH. In all experiments with rats in which ketosis has been reported, comparatively huge doses of ACTH were injected so that it is readily conceivable that the ketogenic effect of a contaminant might supersede any possible inhibitory effect of adrenal steroids. In Kinsell's experiments in man, smaller and evenly spaced doses of ACTH were used so that the ACTH effect might be anticipated to predominate. Under these circumstances fasting ketosis was inhibited.

The mechanism of the inhibitory effect of cortisone and hydrocortisone on ketosis is not immediately apparent. Admittedly, liver glycogen levels are elevated for a time during adrenal steroid therapy, a situation which is generally correlated with antiketogenesis. On the other hand, these steroids have been considered to be "diabetogenic" and as such might be anticipated to stimulate ketogenesis despite their tendency to increase liver glycogen. Under certain circumstances, it would appear that they are capable of doing just that, as indicated by the report of Ingle *et al.* (1951a) of sporadic glycosuria and ketonuria in cortisone treated rats force-fed a high fat diet. The mechanism of the diabetogenic action of the 11-oxysteroids has not yet been established, although there are data indicating that they may exert both an inhibitory effect on fat synthesis from carbohydrate (Welt and Wilhelmi, 1951; Balmain, Folley and Glascock, 1952) as well as a stimulating effect on glucose production from noncarbohydrate sources (Welt, Stetten, Ingle and Morley, 1952). Speculation as to how these steroids inhibit ketosis would seem premature at this time.

The stimulating effect of cold exposure on fasting ketosis and on adrenal weight is clearly apparent in these experiments and is in agreement with other studies on the effects of nonspecific stress previously noted. Sargent and Consolazio (1951) have recently reported ketonuria in normal human subjects abruptly transferred from a warm to cold climate and performing work under bivouac conditions. Although exercise, psychological stress and cold exposure were all factors in this response, the authors considered the cold exposure as the most important and interpret the ketonuria as an expression of the general adaptation syndrome. We have not been able to find any other reports of ketosis during cold stress, although the development of fatty liver is well known to occur under these conditions (Levin and Farber, 1952). In the present study, blood sugar levels during fasting in the cold were the same as in the unstressed animals during the first 48 hours and thereafter they were higher; yet despite this, ketonemia was greater than the control on day three. Without exact knowledge of liver

glycogen levels, it is not possible to draw any conclusions about the relationship between ketosis and liver glycogen in this experiment. Mirski *et al.* (1938), however, have found that liver glycogen is depleted more rapidly during cold exposure and fasting than during fasting alone. On the other hand, in a previous study in this laboratory, liver glycogen levels following a glucose load after 48 hours fast at 4° C. were found not to be different from those of animals fasted at room temperature (Engel, 1951).

Cortisone, hydrocortisone and ACTH all proved effective in reducing the ketosis of cold exposure, making it seem unlikely that this type of ketosis can be mediated by hypersecretion of adrenal hormone. However, this interpretation must be accepted with caution since there is evidence that only small amounts of the above steroids may be secreted by the rat adrenal cortex (Bush, 1953). It would be desirable to repeat this experiment with corticosterone. Furthermore, ACTH was somewhat less effective than cortisone and hydrocortisone, although the interpretation of results with this agent are complicated by the possibility of contamination of this material with growth hormone, as noted above.

Blood sugar levels in the hormone treated groups were in general higher than in the untreated cold exposed animals and some tended to be lower than in the corresponding unstressed groups. Insofar as these blood values might reflect liver glycogen levels, they are consistent with the generally accepted concept of an inverse relationship between liver glycogen and ketogenesis.

The failure of chronic cortisone treatment to prevent cold-stress ketosis as effectively as the other schedules of treatment is not readily explained. However, it should be noted that this group had a poor survival and a high incidence of infection. The latter might have served as an additional stressful stimulus for ketosis.

The absence of ketosis during fasting in rats which had been exposed to cold for one month prior to fasting and had developed marked adrenal hypertrophy might be taken as further evidence against adrenal hypersecretion being a mediating factor in fasting ketosis. However, there are several alternative possibilities which must be investigated before such an interpretation can be entertained. These animals lost a considerable amount of weight during the adaptation period and hence may have had inadequate fat stores to call upon as a source of ketone precursors. Furthermore, these animals were in a debilitated condition and withstood the fast poorly. Blood samples were obtained with difficulty, suggesting that they may have been suffering from some degree of peripheral circulatory failure, a circumstance under which the ketosis of fasting as well as that from intravenously infused sodium octanoate has been shown to be de-

pressed (Engel and Hewson, 1953). Finally, the mere presence of large adrenals is no assurance that the glands had a high secretory capacity. Indeed, the tendency to hypoglycemia during fasting in these animals might suggest that they were relatively adrenal insufficient. These and other points concerning the metabolism of ketones in the cold adapted rat will be the subject of future communications.

On the whole, it would seem reasonable to conclude from these studies that the action of the adrenal cortex in ketone metabolism is in the direction of suppressing ketosis rather than stimulating it, at least during fasting and cold stress. Whether other hormones such as growth hormone or thyroxin are more directly concerned in the ketosis of fasting or stress remains to be established. The demonstration that adipokinin may be found in the urine of fasting rabbits (Weil and Stetten, 1947) and the R.Q. depressing factor in blood of fasting animals and man (Recant, 1952) lends support to the thought that a pituitary factor is involved in fasting ketosis. Szego and White (1947) have already suggested that growth hormone secretion may be increased during fasting. This subject is presently under investigation in this laboratory.

SUMMARY

1. Treatment of normal rats with 5 or 10 mg. of cortisone acetate or hydrocortisone acetate daily inhibits ketosis and increases the blood sugar during a five day fast at 25 to 27° C.

2. Four mg. of ACTH daily had no effect on fasting ketosis, although increasing the blood sugar and adrenal weight.

3. Fasting during cold exposure at 4° C significantly increased ketosis above that from fasting at 25 to 27° C.

4. Treatment of cold-exposed rats with 5 or 10 mg. of cortisone acetate or hydrocortisone acetate or with 4 mg. of ACTH daily depressed cold-stress ketosis, but did not reduce it to the same low level as hormone treatment in unstressed animals. Blood sugar levels were elevated over untreated cold stressed rats, but tended to be somewhat lower than in hormone treated control rats.

5. Acclimatization to 4° C. for one month prevented ketosis on a subsequent fast and blood sugar levels were maintained less well on fasting in acclimatized than in unadapted rats.

6. It is concluded that the over-all effect of adrenal hormone action is to inhibit ketosis.

Acknowledgements

We are indebted to Dr. Elmer Alpert of the Merck and Co. for generous supplies of cortisone acetate and hydrocortisone acetate and to Dr. Irby Bunding of the Armour Laboratories for the ACTH used in these experiments.

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STEROID DIABETES IN GUINEA PIGS

EFFECTS OF CORTISONE ADMINISTRATION ON BLOOD- AND URINARY GLUCOSE, NITROGEN EXCRETION, FAT DEPOSITION, AND THE ISLETS OF LANGERHANS

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THE 11-oxygenated corticosteroids and ACTH have the well known property of aggravating an existing diabetes in humans and in rats, dogs and cats. In normal rats, however, a steroid diabetes can be induced only by simultaneous force feeding (Ingle, 1941; Ingle *et al.*, 1946; Ingle *et al.*, 1951).

This study presents a report on cortisone diabetes in guinea pigs with free access to food. This diabetes resembles in many respects Cushing's syndrome in man but differs from the steroid diabetes in force fed rats. The most striking observations in the cortisone treated guinea pig were an extraordinary hypertrophy of the islets of the pancreas, increased gain of weight, obesity, and a positive or slightly positive nitrogen balance.

MATERIALS AND METHODS

Male guinea pigs weighing about 600 gm. were used. All animals were fed Purine rabbit chow checkers and greens daily. After the body weight was followed for 3 to 4 weeks prior to the experiment, the animals were placed in individual metabolic cages. From that time on they received 50 gm. of lettuce daily, equivalent in protein content to 3 gm. rabbit checkers, and an ad lib. weighed amount of rabbit checkers. The amount of food consumed was determined by subtraction. During the first 3 to 6 days in the metabolic cages almost all guinea pigs lost weight. After the animals had regained their previous weight, analyses were run for about 8 days prior to the administration of cortisone acetate.

In some animals, one of the testicular fat bodies was then removed, together with the testis and the scrotal sac. After regaining the pre-operative body weight, the cortisone injections were started. The steroid was injected subcutaneously during each morning feeding. It was injected in two equal daily doses, the second dose being given in the late afternoon.

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Changes in the total amount of adipose tissue were estimated by comparison of the body weight with the weight of the testicular fat bodies. These fat bodies, present in many rodents, can easily be dissected, weighed and analyzed. In the same animal each one of the two has approximately the same weight, the weight difference being usually not more than 10%, very rarely up to 20%. Removal of one of the fat bodies does not affect the normal growth and fat deposition in the remaining one. During periods of normal weight gain, the weight of the testicular fat bodies increases more than the body weight. Of course, during starvation the weight of these fat stores decreases more than the body weight. Weight changes of the fat bodies out of proportion to changes in body weight indicate an increased deposition or mobilization of fat (Hausberger, 1937; Hausberger and Neuenschwander-Lemmer, 1937). As analyzed by Hausberger and Guyot (1937) the content of total lipids, water and nitrogen of each fat body of one animal is essentially the same. In the following description we will refer to the acetone-petrolether extracted lipids as "fat" and to the difference between wet weight of the fat body and fat plus dry residue as "water." Fat bodies of normal guinea pigs consist 85 to 95% of fat, 4 to 13% of water and 1 to 2% of fat free dry residue. This dry residue consists of 80 to 95% protein.

The animals had free access to food containing 21% protein in dried form which was mostly not digestible. To avoid the determination of the nitrogen excretion in feces, and assuming that a certain constant percentage of nitrogen is always excreted with the urine, the nitrogen balance was estimated in the following way: Urine was collected every 24 hours, acidified with citric acid, and preserved with thymol. The amount of nitrogen excreted was divided by the amount of food consumed during this period, and the resultant value will be called the *nitrogen food ratio.* Normally, an increase of food consumption results in a corresponding increase in the excretion of urinary nitrogen. The ratio will remain the same if the food is of uniform composition. Increase of the ratio indicates an excess excretion, a decrease a retention of nitrogen.

Blood was obtained by cardiac puncture. The following methods of chemical analysis were used: urinary glucose, Benedict (1911); urinary and adipose tissue nitrogen, micro-Kjeldahl method; blood sugar, Durham, Bloom, Lewis and Mandel (1950); urinary acetone, Dumm and Shipley (1946).

For histological evaluation of the beta cells, the pancreas was stained according to Gomori (1941).

EXPERIMENTS AND RESULTS

Five guinea pigs received 2.5 mg. cortisone acetate daily for 2 to 40 days. This amount clearly did not alter the blood sugar level but did produce a significant stimulation of the islets of the pancreas. The injection of 10 to 50 mg. of cortisone acetate produced a steroid diabetes in 17 animals. In 9 of these animals food intake and urinary nitrogen were determined. The metabolic changes induced by diabetogenic amounts of cortisone acetate were, with minor variations, the same in all animals and can be seen in Figure 1.

Body weight. (Figs. 1 and 2) all animals gained weight during the control period. The administration of 2.5 mg. cortisone daily produced a moderately increased gain of weight for one week in one out of the 5 guinea pigs, thereafter the weight gain became normal and possibly a little re-

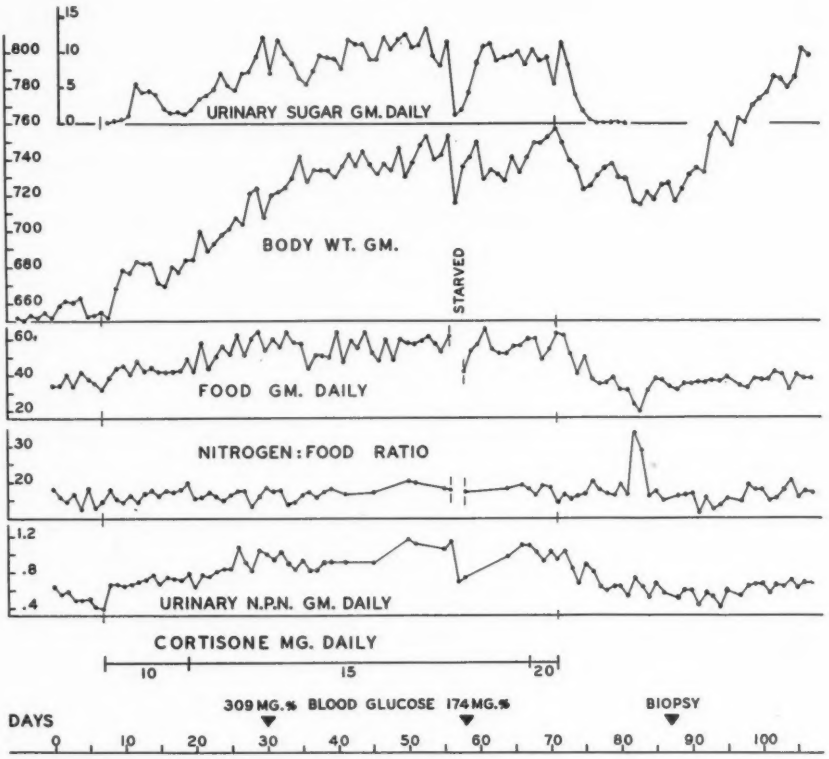

FIG. 1

tarded. This animal was sacrificed after 40 days and was found to be very fat. When 10 mg. or more were administered, there was always a considerably augmented gain of weight. After one to four weeks of treatment, however, the weight gain became less marked but upon increase of the cortisone dosage a new increase of the body weight for a limited period of time was produced. The injections were discontinued in 4 guinea pigs after 16 to 65 days of treatment. All these animals then lost weight. One or two weeks later they started to regain weight, all of them more rapidly than in the control period.

Guinea pigs sacrificed after 2 or more weeks of treatment always showed more adipose tissue than normal controls. Four animals that received cortisone for 4 weeks and longer were found to be extremely obese, the fat stores were enlarged, and adipose tissue had developed in places where normally hardly any is present. The lobules of the pancreas, normally

lying close together (Figs. 3 and 5), were separated by adipose tissue as were also the acini (Figs. 4, 7, 8 and 9). The atrophic tissue of the thymus was almost completely replaced by enlarged amounts of adipose tissue (Fig. 10). The testicular fat bodies of these animals were very large and heavy. In two animals, one receiving cortisone for 40 days and the other one for 42 days, one testicular fat body was removed prior to the beginning of the experiment. Their weight was at this time 1420 mg. or 2.1% of the body weight, and 1320 mg. or 2.0% respectively. When these animals were sacrificed, the weight of the remaining fat bodies had increased to 3040

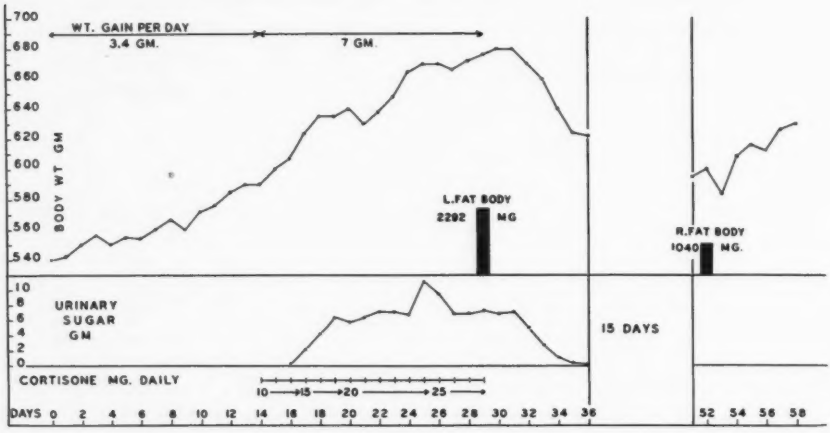

FIG. 2

mg. or 4.6% and 3550 mg. or 4.6%, respectively. This weight increase out of proportion to that of the body weight reflects the general obesity of such animals. In one other animal (Fig. 2) one of the two fat bodies was removed when the cortisone injections were discontinued. The guinea pig which had gained weight disproportionately during the steroid administration, now lost weight. Twenty-three days later, after the animal had regained weight, the second fat body was removed. Its weight was considerably reduced. It therefore seems apparent that the weight gain during the period of cortisone administration is mostly due to an augmented fat deposition, and that the loss of weight after discontinuing the injections is caused by a fat mobilization. The weight gain following this latter period probably reflects an accelerated natural growth. The testicular fat bodies of the cortisone treated guinea pigs always showed a very high fat content, above 90 to 95% of the wet weight. Although this percentage is still within the aforementioned range, it shows clearly that the weight increase of the adipose tissue is due to a true fat deposition.

Urinary glucose. The administration of up to 5 mg. of cortisone for 1 to 2 weeks, induced a glucosuria in none of the guinea pigs. When the dosage was raised to 10 mg., 3 out of 6 animals excreted within 4 to 6 days after start of the injections, up to 1.5 gm. of sugar daily. This glucosuria showed a tendency to diminish and to disappear. Five animals received initially 10 mg. of cortisone daily and in all of them a rising glucosuria could be observed with a maximum of 5.5 gm. of sugar per day. All of these animals, also, showed a decreasing glucosuria after about one week. Therefore, it was not surprising to observe that in the 2 guinea pigs where the cortisone dosage was not increased for 10 days the glucosuria disappeared. An increase of the dose, however, was with exception of one animal able to overcome this tendency. Fifteen to 25 mg. of cortisone daily caused a considerable glucose excretion. The peak was usually reached after 10 to 15 days and a rather constant glucosuria of 8 to 12 gm. per day could be maintained with this dosage until discontinuation of the administration or until the animals were sacrificed. The maximum glucose excretion in one animal was 17 gm. per day. In one guinea pig the glucosuria disappeared almost completely within two weeks, despite the increasing of the dose to 30 mg. daily. In 2 animals the glucosuria was observed for 9 and 10 weeks, respectively, and another has now been diabetic for more than 7 months.

When food was withheld for 24 hours, the urinary sugar diminished considerably (Fig. 1). A rise of the cortisone dosage produced a temporary elevation of sugar excretion in most animals. A direct relationship between cortisone dosage and amount of urinary sugar could not clearly be established in the 16 diabetic guinea pigs. At the peak of the glucosuria, 150 to 280 cc. of urine per day were excreted.

Blood glucose. In 8 animals blood sugar determinations were made during the first days of cortisone administration while the urinary glucose concentration was between 0.2 and 0.4%. On two occasions, the blood glucose was 140 mg. and 155 mg.%, suggesting that cortisone may lower the kidney threshold for sugar. When the glucosuria was fully developed the blood glucose rose to high diabetic levels. The highest value determined was 435 mg.%. After 24 hours of fasting the blood glucose decreased in one animal from 309 to 174 mg.% (Fig. 1), in another from 322 to 168 mg.%. Both had been diabetic for weeks. All guinea pigs receiving 2.5 mg. of cortisone daily had non-fasting blood glucose levels of from 115 to 135 mg.% which was similar to our controls. This compares with Charalampus and Hegstedt (1949) who have given 85 to 150 mg.% as the normal range.

Urinary non protein. The daily urinary excretion was followed in 9 guinea pigs with marked diabetes. In three animals it did not rise out of proportion with the food consumption. These animals received during the

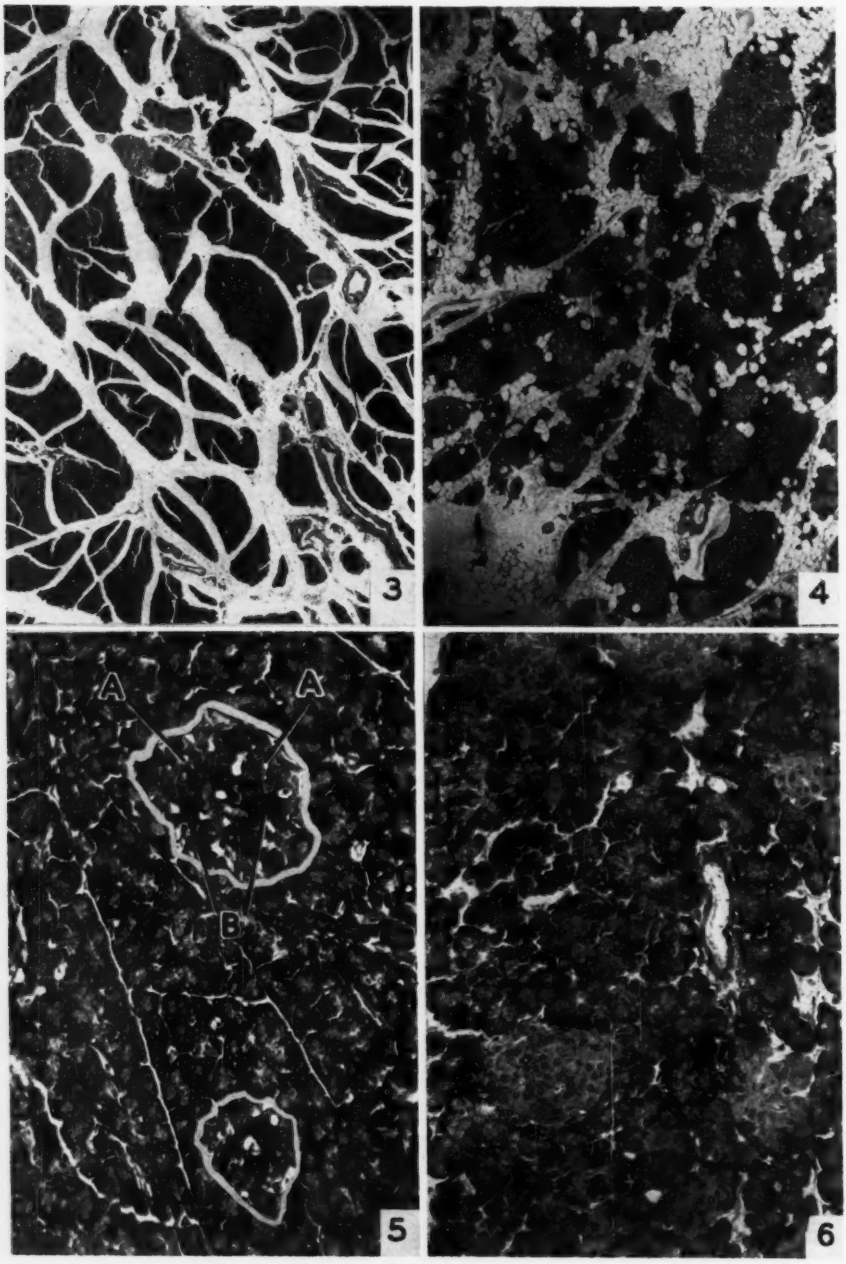

PLATE 1

first three days 5 mg. of cortisone. This dose was slowly increased to 20 and 25 mg. until a marked glucosuria was maintained. In three others the nitrogen food ratio presented a curve similar to that in Figure 1, although possibly there was an insignificant rise. The animal in Figure 1, during the 8 days prior to the experiment proper, excreted 1560 mg. of urinary nitrogen per 100 gm. of food consumed, while during the first 16 days of the cortisone administration this value was 1680 mg. of nitrogen per 100 gm., and during the last 8 days after discontinuing the injections it was 1700 mg. nitrogen per 100 gm. of food. All these animals received 10 to 25 mg. of cortisone daily. Two guinea pigs were injected with 20 mg. and 30 mg. of cortisone per day throughout the entire experiment. The nitrogen excretion was followed for approximately 3 weeks. Two to three days after start of the injections the nitrogen food ratio rose about 25%. This nitrogen loss was not maintained, however, and the nitrogen food ratio returned within the next 2 and 3 days to a value only slightly above that prior to the experiment proper. The same nitrogen balance behavior was seen in one animal which received 50 mg. of cortisone per day for 15 days.

Urinary acetone. About 45 urine samples were examined for acetone but no acetonuria was observed.

Adrenals, lymph nodes and thymus. The average weight of the adrenal glands of 12 controls were .053% of the body weight. The adrenals of all animals receiving 5 mg. or more of cortisone acetate showed a definite atrophy of the cortex. Three animals, however, treated for 4 weeks and longer, had adrenals of almost normal weight. This observation suggesting an adaptation to cortisone administration is being investigated in

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PLATE 1. EXPLANATION OF FIGURES

Photomicrographs of sections of the pancreas of guinea pigs, fixed in Bouin's fluid, sectioned at 4μ and stained with Gomori's chrome hematoxylin and phloxine. A, alpha cells; B, beta cells.

3. Pancreas of a normal guinea pig. Note the presence of areolar tissue and the absence of adipose tissue between the lobules. Only a few islets are visible. ×50.

4. Pancreas of a cortisone treated animal severely diabetic for 73 days (blood sugar, 334 mg. %; maximum urinary sugar, 16.5 gm. per day). Note sharp increase in adipose tissue and in number and size of islets. ×50.

5. Normal pancreas showing two islets. The density of the islet cells indicate normal granulation. Note especially that the beta cells are not enlarged (compare Figs. 6, 7 and 8). ×170.

6. Pancreas of a guinea pig that received 2×10 mg. of cortisone daily for 18 days and was moderately diabetic for 14 days. The blood sugar was 204 mg. %, maximum urinary sugar was 3 gm. per day. Eight areas of new islet formation can be seen, each consisting apparently of enlarged, activated, and degranulated beta cells. Such areas occasionally contain mitoses. ×170.

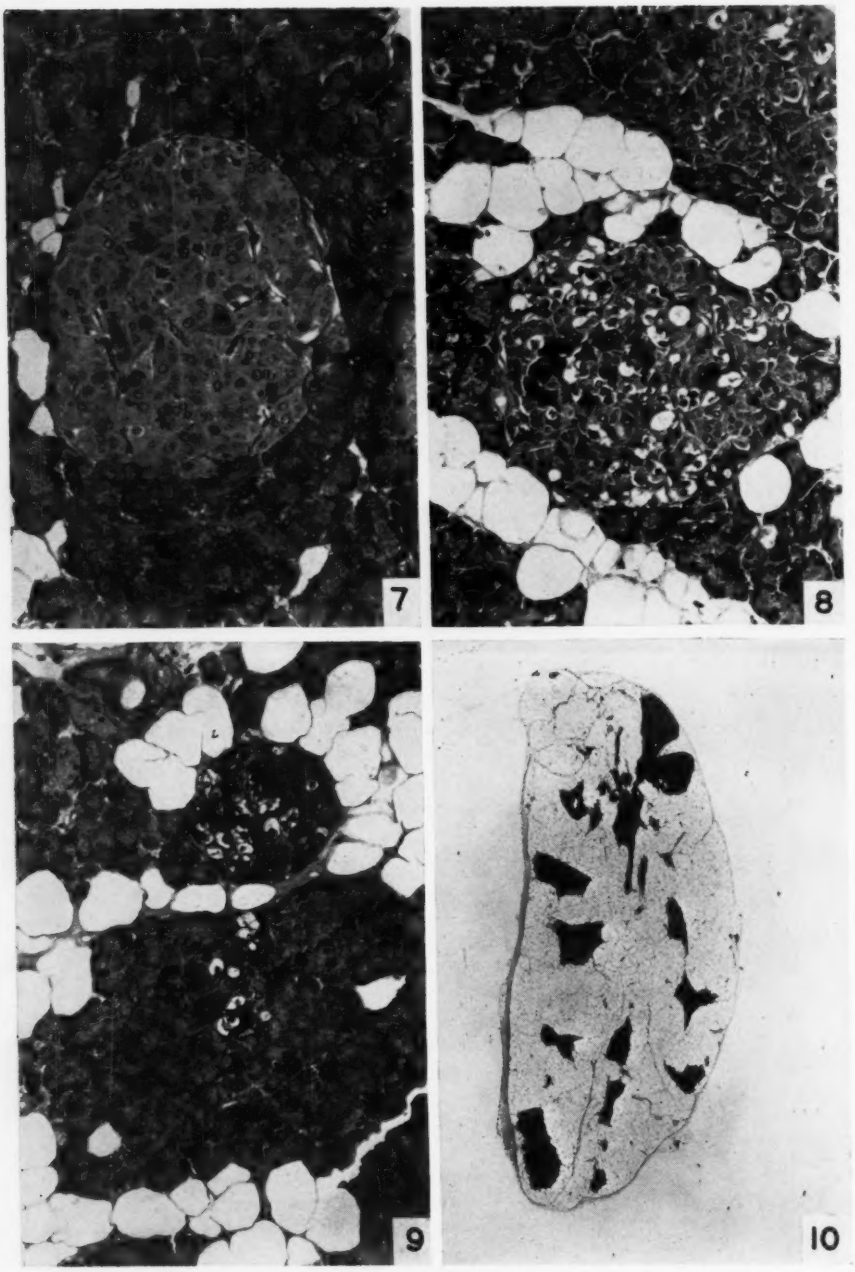

PLATE 2

additional guinea pigs and in rats, and a detailed report will be presented. Lymph nodes and thymus were atrophic. In the latter organ, the specific tissue was replaced to a large degree by adipose tissue (Fig. 10), although the gross weight of the thymus did not change significantly.

Pancreas. A detailed report on the histological findings in the islets will be published elsewhere. As mentioned previously, the most striking observation in all cortisone treated animals was an extraordinary hyperplasia and hypertrophy of the islets (compare Figs. 3 and 4, and 5, 6 and 7, respectively). Islets increased in number by cell enlargement and by a marked new formation of beta cells from the duct system, from centroacinar cells and, according to others (Woerner, 1938) from acinar cells (Fig. 6). The newly formed smaller islets consisted almost entirely of beta cells (Fig. 6) while in the greatly enlarged older islets, the number of alpha cells seemed markedly decreased, at least relatively (Figs. 7 and 8).

Five guinea pigs received 2.5 mg. of cortisone daily for from 2 to 5 days. The islets showed enlargement after two injections and the beta cells showed a normal granulation. After 4 to 5 injections, however, enlargement of the capillaries of the islets, partial degranulation and marked hyperplasia and hypertrophy of the beta cells could be seen. One animal continued to receive 2.5 mg. of cortisone daily for 40 days. The total islets volume after this time had very sharply increased and the formation of new beta cells was considerable. The granulation of the beta cells was possibly less and there was no enlargement of the capillaries. The blood sugar levels of these animals were normal, as already mentioned. It is significant to note that similar changes could be produced by administration of growth hormone in an amount which did not elevate the blood sugar level (Hausberger and Ramsay).

PLATE 2. EXPLANATION OF FIGURES

Figures 7, 8 and 9 are photomicrographs of the pancreas of guinea pigs, fixed, sectioned and stained as in Plate 1. Figure 10, cervical thymus, was fixed in Bouin's fluid, sectioned at 6μ, and stained with hematoxylin and eosin.

7. An islet from the animal analyzed in Figure 2, removed by biopsy on the seventeenth day of cortisone treatment. Note enlargement of islets as well as of beta cells. A few alpha cells may be seen in the center of the islet. ×170.

8. The same pancreas as in Figure 4, showing 3 islets. Beta cells appear smaller, and many show hydropic degeneration. A few alpha cells may be seen. ×170.

9. Pancreas of the animal analyzed in Figure 1, obtained by biopsy 17 days after discontinuing the cortisone injections. Islets have decreased in size, the cells show normal size and granulation (compare Figure 5) but hydropic cells have not recovered. ×170.

10. Cervical thymus of the guinea pig shown in Figure 4. Note extreme involution of thymic tissue and its replacement by adipose tissue. Normally adipose tissue occupies about 15% of the total volume at this age. ×20.

Guinea pigs receiving larger amounts of cortisone showed similar but more marked changes in the pancreas. Degranulation and hypertrophy of beta cells and new formation of beta cells and islets seemed to be in direct proportion to the duration of the cortisone treatment and to the duration and degree of the steroid diabetes. One animal, for example, received cortisone for 40 days. The first week it received 15 mg. per day but did not develop diabetes. The dosage was increased to 20 mg. the second week and it became markedly diabetic, excreting up to 8 gm. of glucose daily. After the second week, although the dosage of 20 mg. was maintained, the sugar excretion diminished until finally in the last two weeks of the treatment only small amounts (0.4% and up to 1 gm. of glucose per day) were excreted. The hypertrophy of the islets was very pronounced, the loss of the beta granulation, however, was less distinct than in other animals receiving a similar dose but showed a more severe diabetes continuously during the time of the treatment. Especially noticeable were the changes in the pancreas of guinea pigs maintained severely diabetic for 4 weeks or longer. In an animal which received cortisone for 84 days and was diabetic for 73 days, approximately one third of the total pancreas consisted of beta cells (Fig. 4). Almost all beta cells were apparently completely degranulated and many exhibited hydropic degeneration; that is, they were pale, vesicular, swollen and contained pyknotic nuclei or the remains of nuclei (Fig. 8). Numerous areas of new formation of beta cells and islets seen in each lobule contained apparently fewer hydropic cells than did the older, larger islets. Most probably, a similar histological picture was present in the animal analyzed in Figure 1, before the cortisone injections were discontinued. In this animal a biopsy of the pancreas was performed 17 days after having discontinued the cortisone administration. The size and number of beta cells as well as of islets were considerably diminished. Many of the beta cells had regained granulation, although to a lesser degree. The hydropic beta cells, however, were not able to recover (Fig. 9). Two other animals that had been diabetic for 8 and 12 days, respectively, were sacrificed 7 days after discontinuing the cortisone administration. The pancreas of these animals showed numerous isolated beta cells and a great many small and large islets. The beta cells here, however, were of normal size and contained an abundant amount of granules.

DISCUSSION

The metabolic changes produced by cortisone in the guinea pig resemble in some aspects Cushing's syndrome in man. There was an augmented weight gain, due to an increased fat deposition, especially pronounced when diabetogenic dosages of cortisone were administered. The food consumption rose markedly, in most animals even before the glucosuria

appeared. Discontinuation of cortisone injections was followed by a slow disappearance of the glucosuria, and a loss of body weight, due to the loss of excessive fat. Moreover, there are reports in the literature on the hypertrophy of the islet apparatus in Cushing's syndrome in glucosuric cases as well as in aglucosuric ones (Cushing and Davidoff, 1927; Lukens *et al.*, 1937; Andersen and Haymaker, 1944).

Hypertrophy of the islets and the new formation of beta cells in the guinea pig is so marked that it can not be missed. One may ask why pathologists do not more often report on hypertrophy of the islets in Cushing's syndrome. Obviously the human pancreas does not react in such an extraordinary way. Besides, one has to consider that the routine methods employed by the pathologists do not reveal an increase of the islet volume, unless it is an extraordinary one.

The exhaustion of the beta cells which occurs after subtotal pancreatectomy (Allen, 1922), after administration of anterior pituitary extract (Richardson and Young, 1938) or prolonged glucose injections (Dohan and Lukens, 1948) is generally considered to be caused by overwork of the islets. In the dog and cat the last stage preceding the final destruction of the beta cells is hydropic degeneration of these cells. In guinea pigs maintained severely diabetic for several weeks, many dead and dying hydropic beta cells could be seen. Experiments in partially pancreatectomized guinea pigs are in progress and will show whether the extraordinary regeneration power of the islets in these animals can finally be exhausted.

Cortisone doses in an amount which does not elevate the blood glucose level causes a partial degranulation and a hypertrophy of the islets. It was shown that the secretion of insulin by the islets is not only regulated by the blood sugar level but also by the amount of insulin present in the blood (Hausberger and Ramsay, 1952). One may therefore consider the possibility that cortisone causes an augmented utilization or destruction of insulin and therewith a stimulation of the beta cells.

The hyperglycemia caused by larger doses of cortisone contributed much to the degranulation, hypertrophy and hydropic degeneration of the beta cells.

The short negative phase of the nitrogen balance, clearly present only when very large amounts of cortisone are administered, suggests a rapid adaptation of the protein metabolism to cortisone. Such an adaptation occurs much slower in Cushing's syndrome (Albright, 1943) and in the rat (Ingle, 1949; Ingle *et al.*, 1951). No significant negative phase of the nitrogen balance could be observed when the cortisone dosage was increased slowly, suggesting a shift of the proteins of the atrophic tissues to the growing adipose tissue.

Since excess of insulin enhances fat deposition and synthesis (Drury,

1940; Stetten *et al.*, 1944, 1946; Chernick and Chaikoff, 1950), one may assume that the increased appetite, the heightened production of adipose tissue and the augmented fat deposition as well as the hypertrophy of the islets, as described in the present study, strongly indicate an elevated insulin secretion. This increased secretion may explain the relatively slight disturbance in the nitrogen balance. Experiments in progress using rats, similar to those of Ingle *et al.* (1945), demonstrate that the administration of very large amounts of protamin zinc insulin is able to diminish the excessive nitrogen loss during cortisone administration, and produce at the same time an increased food consumption and a marked obesity in such animals.

The similarity of hyperadrenocortinism in the human and guinea pig, and the difference between guinea pig and rat to cortisone administration may be caused by still other factors. The guinea pigs received a hormone produced normally in large amounts by their own adrenals, which may be the reason for this specific reaction. On the other hand, there is no indication that an excess of the special steroid produced by the adrenals of a specific animal causes the typical picture seen in human hyperadrenocortinism, as shown by Ingle and coworkers (1951). This research group administered ACTH by continuous infusion to forcefed normal rats with the result that the symptoms were essentially the same as in rats receiving cortisone or hydrocortisone. In the guinea pig, with food *ad lib.*, continuous ACTH infusions induce a steroid diabetes with obesity, and so do injections of hydrocortisone (Hausberger and Ramsay, in preparation).

SUMMARY

1. Male guinea pigs receiving 2.5 mg. of cortisone acetate daily show after several days a partial degranulation of the beta cells and a considerable hypertrophy of the islets.

2. Ten to 50 mg. of cortisone daily produce in all animals an increased gain of body weight due to an augmented fat deposition, and a glucosuria and hyperglycemia. The nitrogen balance is positive, or only slightly negative. The islets of the pancreas show an extraordinary hypertrophy. The number and size of beta cells is considerably increased, and loss of the beta granulation takes place. If a severe diabetic condition is maintained for more than 4 weeks, a marked hydropic degeneration of many beta cells can be seen.

3. After discontinuing the cortisone administration, the body weight decreases due to loss of fat and the diabetes disappears slowly. The formerly enlarged and degranulated beta cells regain their normal size and appearance but the severely hydropic beta cells do not recover.

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ENDOCRINE INFLUENCES ON RENAL COMPENSATORY HYPERTROPHY[1]

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THE degree of compensatory hypertrophy following unilateral nephrectomy in the rat is affected by the age of the animal at the time of the uninephrectomy and by the protein intake (MacKay, MacKay and Addis, 1932; MacKay, Addis and MacKay, 1938). Increased excretory work produced by unilateral ureteroduodenostomy was not found to bring about increase in size of the other kidney (Block, Wakim and Mann, 1953). It has been reported in the dog (Winternitz and Waters, 1940; White, Heinbecker and Rolf, 1941) and in the rat (McQueen-Williams and Thompson, 1939; Colonge, 1944) that in the absence of the anterior hypophysis compensatory renal hypertrophy does not occur. In these reports the degree of compensatory hypertrophy was determined by comparing the weight of the remaining kidney at autopsy with that of the excised kidney of the same animal. Fontaine and Veil (1946) and Braun-Mendendez and Houssay (1949) questioned this procedure on the grounds that following hypophysectomy kidney weight diminishes, and if one compares the remaining with the excised kidney the compensatory hypertrophy is masked by the regression in kidney weight which would have taken place had both kidneys remained intact. They compared the remaining kidneys in uninephrectomized hypophysectomized rats with control kidneys of rats that had been hypophysectomized for the same length of time but not uninephrectomized. Both groups found that renal compensatory hypertrophy could still occur after hypophysectomy, although it was apparently somewhat less than in normal rats.

There is agreement (Franseen, Brues and Richards, 1938; Higgins and Ingle, 1939; Canzanelli, Guild and Rapport, 1949) that the rate of liver regeneration following partial hepatectomy is lower in hypophysectomized rats than in normals, with some uncertainty as to how nearly this difference can be explained on the basis of lessened food intake.

The present work, (a) compares the extent of renal compensatory hypertrophy in uninephrectomized hypophysectomized rats with that in normal rats, and, (b) determines the effect on compensatory hypertrophy in hy-

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pophysectomized rats of the daily administration of growth hormone,[2] of thyroxin,[3] or of growth hormone plus thyroxin during the interval between uninephrectomy and autopsy.

METHODS AND RESULTS

All rats used were of the Sprague-Dawley strain obtained from the same source and maintained on an *ad lib* diet of horsemeat, white bread, apples, Purina dog chow and evaporated milk with 1 to 1.2 gm. sugar/100 ml. milk. This diet was selected because it is highly palatable to hypophysectomized rats, thus diminishing their death rate and weight loss due to insufficient food intake. The hypophysectomies were done by the Hormone Assay Laboratories in Chicago. Completeness of hypophysectomy was evidenced by a universal failure to gain weight for 55 days or more after hypophysectomy and by adrenal and gonadal atrophy.

Problem (a), the comparison of compensatory hypertrophy in uninephrectomized hypophysectomized and normal rats, was approached in 2 ways. In the first procedure 18 male rats hypophysectomized 30 days previously at 200 to 220 gm. body weight were divided into 2 sets, one of which was subjected to right nephrectomy. Because the re-

TABLE 1. COMPARISON OF EXTENT OF RENAL COMPENSATORY HYPERTROPHY IN UNINEPHRECTOMIZED HYPOPHYSECTOMIZED MALE RATS WITH THAT IN UNINEPHRECTOMIZED NORMAL RATS

	No. rats	BW at time of nephr.	BW at autopsy	RKW at nephr.	LKW at autopsy	½ TKW at autopsy	$\frac{\text{RKW}}{100\ \text{BW}}$ at nephr.	$\frac{\text{LKW}}{100\ \text{BW}}$ at autopsy	$\frac{\text{TKW}}{200\ \text{BW}}$ at autopsy	Per cent comp. hyp.
		gm.	gm.	mg.	mg.	mg.				
				normal						
no nephr.	11	210 ± 10	318 ± 17			954 ± 69			300 ± 19	
r. nephr.	10	204 ± 7	298 ± 14	805 ± 48	1203 ± 77		358 ± 42	404 ± 16		34.7 ± 5.4
				hypophysectomized						
no nephr.	9	204 ± 12	217 ± 11			471 ± 30			217 ± 11	
r. nephr.	9	203 ± 8	205 ± 9	532 ± 37	582 ± 35		260 ± 21	284 ± 16		30.9 ± 5.9

Right nephrectomy 30 days after hypophysectomy. Autopsy 25 days after right nephrectomy.
Values are shown with standard deviations.
BW = body weight in grams; RKW, LKW and TKW = right, left and total kidney weight in milligrams; nephr = nephrectomy; comp. hyp. = compensatory hypertrophy $= \dfrac{\frac{\text{LKW}}{100\ \text{BW}} - \frac{\text{TKW}}{200\ \text{BW}}}{\frac{\text{TKW}}{200\ \text{BW}}} \times 100$. $t = 1.33$, $p = 0.2$

gression in kidney size which follows hypophysectomy is not quite complete in 30 days the kidneys of the nonnephrectomized hypophysectomized rats served as controls for the remaining kidneys of the uninephrectomized set. Twenty-five days after uninephrectomy and 55 days after hypophysectomy the rats were sacrificed, and the per cent renal compensatory hypertrophy was determined as defined in Table 1. In this way the remaining kidney after nephrectomy is compared with what it would have been if there had been no nephrectomy. At the same time 21 normal male rats weighing from 190 to 220 gm. were divided into 2 sets, and 1 set subjected to right nephrectomy. Because the kidneys of these rats are still growing even without uni-nephrectomy, the kidneys

[2] The growth hormone, R491024, was kindly supplied to us by Dr. I. M. Bunding of Armour Laboratories. Its growth potency compared to their standard was 64 ± 33 per cent.

[3] We are indebted to E. R. Squibb & Sons for the thyroxin used in this work.

of the nonnephrectomized set served as controls. All rats, uninephrectomized and nonnephrectomized, were sacrificed 25 days after the uninephrectomies of the operated rats, and the per cent compensatory hypertrophy determined. Table 1 shows that this value in rats hypophysectomized 30 days before uninephrectomy is not significantly different from that in normal rats.

The other approach was to keep rats after hypophysectomy until renal regression in weight had been completed. With such rats the kidney removed at uninephrectomy can serve as control for the remaining kidney. We have previously followed the ratio of kidney/body weight in 49 hypophysectomized rats from 13 to 138 days posthypophysectomy and have found it to level off between the 34th and 60th day. Since the 10 rats of Table 2 had been hypophysectomized 107 to 153 days at the time of uninephrectomy, the excised kidney of each rat served as control for the remaining kidney. The per cent of compensatory hypertrophy with this method as shown in Table 2 is significantly less than that with the normal rats of Table 1 ($t=4.0$, $p<0.01$), and probably less than with the 30 day posthypophysectomy rats of Table 1 ($t=2.18$, $p=0.04$). It seems probable that the longer interval between hypophysectomy and uninephrectomy accounts for the lesser degree of hypertrophy in Table 2 than in the 30 day posthypophysectomy rats of Table 1. No reason is evident for believing that the hypertrophy in hypophysectomized male rats should be greater than in hypophysectomized female rats.

TABLE 2. DIRECTLY OBSERVED RENAL COMPENSATORY HYPERTROPHY IN FEMALE RATS UNINEPHRECTOMIZED 107 TO 153 DAYS AFTER HYPOPHYSECTOMY

No.	BW at nephr.	BW at autopsy	RKW at nephr.	LKW at autopsy	Per cent comp. hyp.
	gm.	gm.	mg.	mg.	
1	132	140	370	486	31.3
2	122	120	325	372	14.5
3	132	143	335	424	26.6
4	108	123	283	367	29.7
5	122	117	296	372	25.7
6	138	136	391	480	22.7
7	146	137	396	460	16.2
8	99	108	250	315	26.0
9	106	113	306	372	21.6
10	112	118	313	417	33.2
Ave.	122	126	327	407	24.5

Autopsy 25 days after right nephrectomy.

$$\text{Per cent comp. hyp.} = \frac{\text{LKW} - \text{RKW}}{\text{RKW}} \times 100.$$

Problem (b), the determination of the effects of hormone administrations on compensatory hypertrophy in hypophysectomized rats, was approached as follows. The per cent compensatory hypertrophy in three groups of hormone-treated male hypophysectomized rats was determined according to the procedure outlined for Table 1, where the nonnephrectomized set of each group served as the control for the uninephrectomized set. In this way allowance was made for the effect of hormone treatment alone on kidney/body weight ratio. Each set of one group received 0.05 mg/100 gm/day of growth hormone (Armour's standard), of another group 0.01 mg/100 gm/day of thyroxin, and of the third group the combination of the above two. All injections were subcutaneous and were given from the time of uninephrectomy to autopsy. The per cent compensatory hypertrophy in each of these groups is seen in Table 3, and may be com-

pared with the values shown in Table 1. Except for an apparently slightly lower value with the group treated by growth hormone alone, the values for the hormone-treated groups do not differ from those of the normal and the untreated hypophysectomized of

TABLE 3. EFFECTS OF HORMONE TREATMENTS ON RENAL COMPENSATORY HYPERTROPHY IN HYPOPHYSECTOMIZED MALE RATS

	No. rats	BW at time of nephr.	BW at autopsy	RKW at nephr.	LKW at autopsy	½ TKW at autopsy	RKW / 100 BW at nephr.	LKW / 100 BW at autopsy	TKW / 200 BW at autopsy	Per cent comp. hyp.
		gm.	gm.	mg.	mg.	mg.				
			growth hormone							
no nephr.	6	203 ± 14	284 ± 17			650 ± 26			229 ± 12	
r. nephr.	8	198 ± 9	269 ± 15	575 ± 47	768 ± 52		290 ± 13	285 ± 9		24.5 ± 4.1
			thyroxin							
no nephr.	6	198 ± 16	183 ± 17			665 ± 45			363 ± 19	
r. nephr.	6	185 ± 11	175 ± 10	567 ± 60	830 ± 106		306 ± 17	476 ± 80		31.1 ± 12
			growth hormone plus thyroxin							
no nephr.	6	124 ± 4	163 ± 6			665 ± 74			407 ± 23	
r. nephr.	4	117 ± 7	124 ± 4	353 ± 33	848 ± 115		298 ± 42	540 ± 57		32.8 ± 13

Uninephrectomy 30 days after hypophysectomy. Autopsy 25 days after uninephrectomy.
Growth hormone, 0.05 mg./100 gm./day (Armour's standard); thyroxin, 0.01 mg./100 gm./day, daily subcutaneously from time of uninephrectomy to autopsy. Abbreviations and calculations as in Table 1.

Table 1. Comparisons of the untreated hypophysectomized group of Table 1 with the growth hormone treated, the thyroxin treated and the growth hormone plus thyroxin treated hypophysectomized groups of Table 3 give p values of $0.035 > 0.5$ and > 0.5 respectively. All rats used in this work were in the age range from 100 to 230 days at time of uninephrectomy, within which range the degree of hypertrophy is independent of age (MacKay, MacKay and Addis, 1932). The influence of age is thus presumably not operating in our groups.

DISCUSSION

Our average renal compensatory hypertrophy of 34.7% in normal rats may be compared with previously reported values of 32, 30.9, and 32.9 in rats 90, 180 and 270 days old at uninephrectomy, respectively (MacKay, MacKay and Addis, 1932), and of 40% in rats of about 100 grams body weight and presumably about 60 days of age (Colonge, 1944). Braun-Menendez (1946) found 60 to 70% hypertrophy in normal rats of 100 to 150 grams, while Braun-Menendez and Houssay (1949) found 36% in craniotomized rats which were unsuccessfully hypophysectomized and which served as controls for their hypophysectomized series. Values up to 100% or higher have been reported in very young (6 to 27 day) rats (Jackson and Shiels, 1927) and in rats on very high protein diets (Smith and Moise, 1927). Direct comparison of the results of different workers is not always possible, since age at time of nephrectomy, interval between nephrectomy and autopsy, and diet have varied. Our values of 31 and 24.5% in hypophysectomized rats in Tables 1 and 2 may be compared with that of 22% without forced feeding and 26% with forced feeding found by Braun-Menendez and Houssay (1949) at 20 days after uninephrectomy. Under our experimental conditions, which were the same for Tables 1 and 3, the hypertrophy was the same (except for the growth hormone treated

group of Table 3) in untreated and treated hypophysectomized rats as in untreated normals. We believe that the procedure outlined in Table 1 makes adequate allowance for average changes that would have occurred without uninephrectomy. The thyroxin treated hypophysectomized group (Table 3) showed no significantly different per cent compensatory hypertrophy from the untreated hypophysectomized group of Table 1 ($p = > 0.5$). The growth hormone plus thyroxin group (Table 3) was not different from the untreated hypophysectomized group of Table 1 ($p = > 0.5$). In view of the finding that growth hormone added to thyroxin did not bring the hypertrophy below that seen with thyroxin alone, we do not consider the finding of a somewhat lessened hypertrophy with the growth hormone treated group as convincing evidence of a real difference from the untreated hypophysectomized group of Table 1.

SUMMARY

The per cent renal compensatory hypertrophy seen 25 days after uninephrectomy was the same (30.9) in rats that had been hypophysectomized 30 days before uninephrectomy as in normal rats (34.7). When an interval of 107 to 153 days was allowed between hypophysectomy and uninephrectomy, the per cent hypertrophy seen 25 days after uninephrectomy was significantly lower (24.5).

With 3 groups of rats treatment with growth hormone, with thyroxin, and with growth hormone plus thyroxin was begun at the time of uninephrectomy 30 days after hypophysectomy. No convincing evidence was obtained that any of these treatments resulted in 25 day postnephrectomy hypertrophy different from that observed in untreated rats which had been hypophysectomized 30 days before uninephrectomy.

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RELATION OF ASCORBIC ACID NUTRITION TO THE *IN VITRO* METABOLISM OF CORTISONE BY SURVIVING LIVER TISSUE

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STUDIES from this laboratory suggested that dietary ascorbic acid excess may prevent the breakdown of injected cortisone to 17-ketosteroids. Adrenalectomized female rats injected with cortisone excrete large quantities of 17-ketosteroids and moderately large amounts of corticosteroids (phosphomolybdic acid reducing lipids, PRL). Simultaneous injection of ascorbic acid along with cortisone prevented the rise in urinary 17-ketosteroids (Bacchus, Altszuler and Heiffer, 1952; Bacchus and Heiffer, 1953), but led to an increase in the PRL excretion (Bacchus and Heiffer, 1953). These findings were associated with a prolongation of the hematologic action of cortisone (Bacchus, Altszuler and Heiffer, 1952b; Bacchus, Heiffer and Altszuler, in preparation). The above data, collected in adrenalectomized animals, suggested that large doses of ascorbic acid prevent the breakdown of cortisone, and in this manner help maintain larger amounts of hormone in the circulation. The present paper reports data on the *in vitro* disappearance of cortisone acetate when it is incubated with liver tissue of ascorbic acid deficient animals, and of animals given massive doses of ascorbic acid.

METHODS

Weighed amounts of liver tissue were incubated with known amounts of cortisone acetate in phosphate saline buffer. At the end of the incubation the amount of steroids present was determined. Two methods of incubation and extraction were used. In the first series (A) the 1 gram (wet wt.) of liver was incubated with 3,000 micrograms of cortisone acetate in 15 ml. phosphate saline buffer (pH 7.43) at 38 C. The incubation procedure lasted three hours, and the preparations were shaken every ½ hour. At the end of the incubation the tissues were separated off, and the incubate extracted twice with equal amounts of a chloroform:ether (1:4) mixture in a separatory funnel. The extract was washed with water, dehydrated with anhydrous sodium sulfate and evaporated in vacuo at 40 C. Preliminary experiments indicated that chloroform ether extraction resulted in 95% recovery of known amounts of hormone, whereas, chloroform extraction (as recommended by Porter and Silber, 1950) resulted in 93% recovery. One advantage of the chloroform-ether procedure, however, is that the evaporation step is more abbrevi-

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ated. The extract was then dissolved in absolute methanol and aliquots of this solution were used for color development.

In the second series (B) of experiments the incubation and extraction procedures were as follows: Weighed amounts of fresh liver tissue (60 mg.) were incubated with 200 micrograms of cortisone acetate in 3 ml. of phosphate saline buffer at pH 7.40. The incubation was conducted aerobically and lasted three hours, the tubes were agitated every hour. At the end of the incubation 7 ml. of chloroform were added to the tissue-and-incubate preparation. The tubes were stoppered and shaken for 90–110 times, then they were centrifuged for three to four minutes. At the end of the centrifugation the chloroform phase was separated from the aqueous (buffer) phase by a thin interphase consisting of liver tissue and an amorphous mass. The aqueous layer was aspirated off. Anhydrous sodium sulfate was then added to the vessel and this was mildly agitated, then centrifuged for five minutes. The chloroform extract was then decanted into a 15 ml. tube, the incubation tube being washed with another 3 ml. of chloroform. The extract was then evaporated to dryness in a vacuum desiccator at 40° C. and under moderate vacuum. The extract was then shaken up in absolute methanol and aliquots were used for color development.

Color Development: The amount of cortisone remaining in the preparations was determined by the method of Porter and Silber (1950) for steroids with the 17-OH, 20, 21-ketol structure. The standard 17-OH, 2-, 21-ketol structure was cortisone acetate.

EXPERIMENTS AND RESULTS

Series 1

In the first series incubation and extraction procedure A was employed.

Experiment 1: Liver tissue of guinea pigs was used. One group of guinea pigs was maintained on Purina Rabbit Chow and tap water. This diet without supplements of ascorbic acid, produces Vitamin C deficiency, and frank scurvy within three weeks (Bacchus, unpublished data). Control animals were pair-fed with Chow and in addition received daily injections of sodium ascorbate equivalent to 1.33 mg. ascorbic acid/100 gm./ day. This amount of vitamin is sufficient to maintain tissue levels and to prevent gross signs of scurvy (Telford, Roe, and Keuther, 1943). This low dosage level was employed in order to avoid large doses which may affect the metabolism of steroids, and yet sufficient to prevent deficiency. (It was observed in this laboratory that this dosage does not alter the urinary 17-KS and PRL or the cytochemical properties of the adrenal when compared to normal animals on a Chow and fresh greens diet.) Liver tissue (one gram wet weight) from control animals and from animals on the deficient diet for five days and for 18 days were compared in their ability to metabolize cortisone. In addition, the influence of ascorbic acid added *in vitro* on this process was also studied. Control incubation vessels contained: liver without steroid; steroid without liver; ascorbate with and without liver. Unincubated controls were also run. The data are presented in Table 1. It is observed that the liver of the control animals metabolized the side

TABLE 1. BREAKDOWN OF CORTISONE ACETATE BY LIVER SLICES FROM ASCORBIC ACID-DEFICIENT, AND CONTROL, GUINEA PIGS*

Type of preparation	Micrograms of cortisone acetate lost/gm.	Per cent lost	P
Normal (control) (5)	1,250 ± 140 (S.E.)	42%	<0.05
Vitamin C-deficient 19 days (5)	1,590 ± 42	53%	
Vitamin C-deficient 5 days (3)	1,630 ± 39	54%	
Vitamin C-deficient 5 days + 100 mg. ascorbate added *in vitro* (2)	1,530 ± 65	51%	

* Each experimental incubation vessel contained 15 ml. of phosphate-saline buffer (pH 7.43), 1 gram of liver slice, and 3000 micrograms of cortisone acetate (suspension). The cortisone remaining after 3 hours of incubation (aerobically) at 38° C. was determined by the Porter-Silber assay. Control vessels in which cortisone was incubated without liver tissue, with and without ascorbic acid indicated 95–102% recovery. Per cent degradation is calculated on the basis of the amount of hormone originally in the preparations, i.e. 3000 micrograms each. The numbers in parentheses indicate the numbers of separate preparations, with tissues from separate animals, used.

chain (Porter-Silber assay) to the extent of 1,250 micrograms for the gram of liver tissue. This is equivalent to 42% of the steroid originally in the preparation. The liver tissue of the 18-day deficient animal metabolized the hormone to the extent of 1,590 microgm./gm. equivalent to 53%, an increase of 27% over the control. It was observed that *in vitro* additions to the preparations did not alter the disappearance of the 17-OH, 20,21-ketol structure.

In the second experiment of this series liver tissues from female rats (200 gm.) were used. Tissues were obtained from normal intact rats on Purina Laboratory Chow and tap water, and from such rats given 200 milligrams of ascorbic acid (Na ascorbate)/100 gm. for the two preceding days—by intraperitoneal injection. Control incubations as described above were also run. The data are presented in Table 2. The rate of disappearance of the 17-OH, 20,21-ketol structure when rat liver is used is similar to that of the control guinea pig. A significantly smaller amount of this structure is lost when liver tissue from animals on dietary ascorbic acid excess are used. It is noted here also that *in vitro* additions of ascorbic acid to the preparations fails to alter the extent of loss of this structure. Unpublished

TABLE 2. BREAKDOWN OF CORTISONE ACETATE BY LIVER TISSUE FROM RATS ON DIETARY EXCESS OF ASCORBIC ACID, AND FROM CONTROLS*

Type of preparation	Micrograms of cortisone acetate lost/gm.	Per cent lost	P
Normal control (7)	1,270 ± 50	42%	<0.05
Excess dietary Vit C. (4)	1,030 ± 40	34%	
Normal control + 100 mg. ascorbate *in vitro* (4)	1,290 ± 42	43%	

* See explanation of Table 1.

studies indicate that the *in vitro* ascorbate may depress the loss of the Δ^4, 3-ketone group of the steroid.

Series 2

In this series the incubation procedure B was used. Larger numbers of animals were used in this experiment. The data are presented in Table 3. It is observed that the *in vitro* loss of the structure by normal liver under these conditions is 110 ± 2.7 μgm/60 mg., equivalent to 55% of the original amount of steroid. *In vitro* addition of ascorbate fails to alter the extent of loss. It may be added that control recovery studies in which cortisone acetate was incubated without liver indicated a 10% loss of the structure. Extracts of liver incubated without steroid revealed very small amounts in the preparation.

TABLE 3. BREAKDOWN OF CORTISONE ACETATE BY LIVER SLICES FROM RATS ON DIETARY EXCESS OF ASCORBIC ACID, AND FROM CONTROLS*

Type of preparation	Microgram of cortisone acetate lost/60 mg.	Per cent lost	P
Normal control (8)	110 ± 2.7	53%	<0.01
Excess dietary Vit. C. (9)	70 ± 2.1	35%	
Normal plus 20 mg. ascorbate *in vitro* (8)	100 ± 5.6	50%	<0.01
Cortisone acetate (200 μgm) with no liver tissue	20 ± 5.0	10%	
Liver tissue with no cirtisone acetate (5)	<10 (7 ± 1.8) micrograms recovered		

* 60 mg. liver plus 200 μgm cortisone acetate incubated aerobically in 3 ml. phosphate-saline buffer (pH 7.40), 38° C. for 3 hr. Loss of 17,21-OH, 20-keto- structures determined by Porter-Silber assay. Numbers in parentheses indicate numbers of tissues, from separate animals, used.

DISCUSSION

The data demonstrate that liver slices are capable of metabolizing the 17-OH, 20,21-ketol structure in *in vitro* preparations. Dietary ascorbic acid excess decreases the ability of the liver to metabolize this structure (17-OH, 20,21-ketol) of cortisone. The failure of addition of ascorbic acid to the *in vitro* preparations to maintain these structures suggests that some vital process in the body is necessary for the action of the vitamin. The nature of this process is not known. The observations in the vitamin-deficient animals suggest that the liver under these circumstances is unable to maintain the 17,21-OH, 20 keto structure. *In vitro* ascorbic acid in this case also fails to alter the reaction.

These *in vitro* observations support the data from *in vivo* studies in this laboratory. It was observed that ascorbic acid increased the urinary corticosteroids and depressed the 17-KS (Bacchus, Altszuler and Heiffer,

1952a) in adrenalectomized female rats given cortisone (Bacchus and Heiffer, 1953). Ascorbic acid treatment was also observed to depress the 17-KS excretion, and to maintain the levels of circulating glucocorticoids (as indicated by lymphopenia and eosinopenia) in adrenalectomized female rats given cortisone (Bacchus, Altszuler and Heiffer, 1952b; Bacchus, Altszuler and Heiffer, to be published).

It had been observed by Kayahan (1952) that massive doses of ascorbic acid given to human subjects is followed by a rise of urinary corticosteroids and a decrease of 17-KS. That author interpreted the data as indicating a shift in the type of hormone produced by the adrenal cortex. It should be emphasized that we have reported a similar shift in the *urinary* steroids in adrenalectomized female rats given exogenous cortisone. It was pointed out that a shift in adrenal production could not reasonably explain these findings. Instead, we ascribed the changes to alterations in the metabolism of cortisone by other tissues, and primarily the liver. These *in vitro* data support the above explanation.

The data that ascorbic acid deficiency may be associated with a diminution in the ability to maintain the 17,21-OH, 20 keto structure support still further data from this laboratory. The urinary steroids, adrenal structure, and blood leukocytes were studied in ascorbic acid deficient female guinea pigs and their pair fed controls. It was observed that in early deficiency (2 to 4 days) there was an increase in the urinary 17-KS, a fall in the urinary PRL, a rise in lymphocytes and eosinophils in the deficient animals. The adrenal cortex structure and cytochemical appearance were entirely normal at this stage, however. These data suggested that the primary influence of the deficiency here was an increase in the conversion of corticosteroids to 17-KS, since no signs of alteration in adrenal functions were observed. In later deficiency, terminating in death in cases, there occurred a progressive decrease in the urinary PRL and 17-KS, and with signs of adrenal damage (hemorrhages, lipid depletion). These latter observations suggested adrenal damage with diminution in adrenal secretory ability in late deficiency. These data were collected in studies which involved daily observations.

The work of Nadel and Schneider (1952) indicates a significant and progressive increase in the urinary corticosteroids in scurvy. Our data do not confirm those findings. Banerjee and Deb (1952) on the other hand suggest adrenal hypoactivity in scurvy on the basis of urinary 17-KS and electrolyte balance (1952, 1952b). Those workers also observed the decrease of 17-KS in later deficiency in agreement with our data, but failed to observe the initial rise (2 to 4 days) since they made only determinations on pooled weekly samples.

It is acknowledged that the conclusions from *in vitro* studies are only

suggestive of *in vivo* processes. These observations receive confirmation from *in vivo* studies which we have published and others which are in press. Studies such as these demonstrate that urinary steroid determinations do not provide adequate indices of adrenal activity. Such data may also indicate changes in liver function, and in functions of other tissues. Indeed, it had been suggested by Sayers (1950) that urinary steroids, *in the absence of other data*, should be rejected as adequate indices of adrenal function.

SUMMARY AND CONCLUSIONS

The *in vitro* metabolism of cortisone acetate by surviving liver tissues was studied. Liver was obtained from animals on normal dietary fares, on ascorbic acid deficiency, and on dietary ascorbic acid excess. The data indicate that the ability of liver tissue to maintain the 17-OH, 20,21-ketol structure (Porter-Silber assay) of the steroid is enhanced ($P < 0.01$) in animals receiving excess ascorbic acid, but is depressed ($P < 0.05$) in ascorbic acid deficiency. *In vitro* addition of ascorbic acid to such preparations fails to protect this structure, and fails to replace the maintenance action to tissues of deficient animals. The data are discussed in relation to some *in vivo* studies. Those studies suggested that ascorbic acid may depress the breakdown of corticosteroids.

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LIVER VITAMIN CONTENT AND ADRENAL CORTICAL FUNCTION

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THAT extracts of the adrenal gland are capable of elevating the blood sugar level and liver glycogen stores has been known for some time (Britton and Silvette, 1932). Moreover, the ability to cause deposition of glycogen in the livers of adrenalectomized rats and mice has provided a useful basis for the assay of corticoid substances (Reinecke and Kendall, 1942; Venning, *et al.*, 1946).

In view of the importance of B-vitamins in carbohydrate metabolism, it seemed desirable to determine whether the effect of the adrenal cortex on glycogen formation in the liver is accompanied by an alteration in the vitamin content of the liver. This approach also seemed of interest in view of the reported implication of pantothenic acid in adrenal function and in cholesterol metabolism (Ralli and Dumm, 1952; Guehring *et al.*, 1952).

METHODS

Adult female Holtzman rats were adrenalectomized under ether anaesthesia through a lumbar approach. The rats were kept on a stock diet with normal saline *ad libitum* for about one week and then they were put on a high protein synthetic diet for 5 days prior to autopsy. Intact female rats of the same age were used as controls.

Twenty-four hours before the intended time of sacrifice, food was removed from the cages. Seven hours before sacrifice, a series of intraperitoneal injections of glucose solution was begun ($\frac{1}{2}$ cc. containing 70 mg./cc.). All animals were injected seven times over a 6 hour period (the first 4 injections at 45 minute intervals, the last 3 at hourly intervals) until a total of 250 mg. of glucose had been given. The rats in one of these groups were each given a total of 3 mg. cortisone acetate in addition to the glucose. The animals were sacrificed 1 hour after the last injection.

Sacrifice was accomplished by intraperitoneal injection of nembutal (0.03 mg./gm. body weight). When the animals were under deep anaesthesia, a 100 mg. sample of liver was removed from each animal and placed between 2 pieces of solid CO_2. The pieces of liver were weighed on a Roller-Smith torsion balance while frozen and immediately dropped into a measured amount of hot 20% KOH. This method of removing the liver minimized postmortem glycogenolysis and eliminated the error in weighing inherent in weighing wet, bloody pieces of liver. In order to keep the interval between the last injection of glucose and sacrifice constant, the total time elapsing from the first to

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the last sacrifice did not exceed 15 minutes. The remainder of the liver was removed, homogenized in a glass tissue grinder and prepared for vitamin analysis.

The glycogen determinations were performed by diluting the digested sample with distilled water to 1 mg./cc. and reacting with 0.16% anthrone in 95% sulfuric acid, after the method of Seifter *et al.* (1950).

A liver homogenate containing 1 gm. fresh tissue in 100 ml. was prepared and aliquots taken for vitamin extraction. Biotin was extracted by autoclaving the homogenate (in 6N sulfuric acid) for 1 hour at 120° C. as described by Wright and Skeggs (1944), and assayed by the yeast growth method of Hertz (1943), on a 1:2000 dilution of the neutralized acid hydrolysate.

Pantothenic and folic acid were extracted by enzymatic hydrolysis using mylase P, Ives and Strong (1946). Folic acid was assayed acidimetrically with *Lactobacillus casei* grown on the medium of Tepley and Elvehjem (1945).

Pantothenic acid was assayed by the *Lactobacillus arabinosus* ATCC 8014 grown on Difco medium (essentially the medium of Skeggs and Wright, 1944).

Riboflavin, nicotinic acid and pyridoxine were extracted by autoclaving the liver suspension in 1/10 N hydrochloric acid for 20 minutes at 120° C. Riboflavin and nicotinic acid were assayed with *Lactobacillus casei* ATCC 7469, as described under folic acid.

Pyridoxine was determined by the growth of *Saccharamyces Carlsbergensis* ATCC 9080 as described by Atkins *et al.* (1943).

RESULTS AND DISCUSSION

The data in Table 1 show that cortisone increased the liver glycogen of adrenalectomized rats from 10.2 mg./gm. to 25.7 mg./gm. in Experiment A and from 7.6 mg./gm. to 21.4 mg./gm. in Experiment B. Normal intact animals with no cortisone or glucose injected showed a value of 6.3 mg./gm.

The 6 B vitamins listed showed no particular change under these conditions.

Our observations are in agreement with those of Draper and Johnson (1953) who found that cortisone treatment of intact rats has no appreciable effect upon the riboflavin content of the liver.

The results of these experiments seem to indicate that there is no gross change in the concentration of enzymes containing these vitamins as a result of alteration in adrenal hormonal level. The possibility that other prosthetic groups might be altered cannot be excluded; nor is it possible to say whether or not the prosthetic groups measured are present in an active or inactive form.

It is possible that such a small proportion of the B-factors present in the liver tissue is involved in these transformations that no significant change in concentration is detected.

SUMMARY

Cortisone with glucose, administered to adrenalectomized rats, increased the liver glycogen concentration to approximately 3 times the value found in such animals injected with glucose alone.

TABLE 1. RAT LIVER GLYCOGEN AND LIVER VITAMIN CONCENTRATION
(Mean values ± standard deviation)

Treatment	Rats[1] Group No.	Body wt. gms.	Glycogen mg./gm.	Pantothenate*	Pyridoxine*	Niacin*	Biotin*	Folic*	Riboflavin*
				Experiment A					
Adrenalectomized +Glucose[3]	8	184 ± 11	10.2 ± 2.2	34.5 ± 6.05	6.63 ± 1.25	163 ± 12.4	.763 ± .419	.663 ± .114	30.6 ± 8.92
Adrenalectomized +Glucose[3] +3.5 mg. Cortisone[2]	8	176 ± 13	25.7 ± 5.4	34.5 ± 4.86	5.89 ± .913	161 ± 17.7	.594 ± .0682	.631 ± .165	29.7 ± 5.06
				Experiment B					
Adrenalectomized +Glucose[3]	10	197 ± 21	7.6 ± 2.1	54.9 ± 3.54	6.84 ± .967	151 ± 12.1	.600 ± .0423	.768 ± .137	22.2 ± 1.88
Adrenalectomized +Glucose[3] +3.5 mg. Cortisone[2]	12	206 ± 14	21.4 ± 5.6	50.2 ± 4.34	6.75 ± .841	134 ± 12.7	.578 ± .0522	.682 ± .0575	21.8 ± 1.41
Intact Controls No treatment	10	198 ± 15	6.3 ± 3.5	65.2 ± 2.23	7.09 ± 1.42	142 ± 6.35	.668 ± .0312	.490 ± .049	25.6 ± 1.20

* Vitamin values in micrograms per gram of fresh tissue.
[1] Female Holtzman (Sprague-Dawley) rats, starved 24 hours before sacrifice.
[2] Cortisone acetate. 3.5 mg. in 3.5 cc. injected at intervals over 6 hours (see text).
[3] 250 mg. in 3.5 cc. injected ½ cc. IP at intervals over 6 hours (see text).

Pantothenic acid, biotin, riboflavin, niacin, pyridoxine, and folic acid concentrations in rat liver were not substantially changed by adrenalectomy or the injection of cortisone. The concentrations of these vitamins in the liver of adrenalectomized rats were found to be the same as corresponding values for normal intact rats.

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THE EFFECT OF THERMAL BURNS ON THE THYROID ACTIVITY OF THE RAT[1]

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INTRODUCTION

DECREASED thyroid activity, measured as I^{131} uptake, has been reported following the administration of various stressing agents such as vaccines (Williams, 1949), epinephrine (Soffer, 1949, 1950), chemical agents (Paschkis, 1950), anoxia and abnormal nutritional states (Van Middlesworth, 1950), ACTH and cortisone (Money, 1950; Reiss, 1950) and early stages of infection with Lieshmania (Stauber and Mauer, 1952). Botkin and Jensen (1952) have shown an early decrease in I^{131} uptake by the thyroid following treatment with epinephrine and thyrotropin. Increased I^{131} uptake following the stress of X-irradiation has been observed by Botkin *et al.*, (1952); whereas, the findings of Bogoroch (1951) indicated that during various types of acute stress I^{131} uptake by rat thyroid is consistently less than that of normal rats.

Studies by Gribble and Peters (1951) suggest that burning may stimulate thyroid activity, but the investigations of Sellers (1950) and You (1950) indicate no changes in thyroid activity following burns, as reflected by BMR. These latter investigators showed further that the thyroid of the rat is not causally related to the increased nitrogen excretion following burns. The studies reported here were undertaken to obtain a direct measure of thyroid activity measured as I^{131} uptake following deep thermal burns in the rat.

EXPERIMENTAL

Animals. All rats employed were adult males, average body weight 196 ± 7 gms. The ration consisted of commercial mixed grain with meat and water, *ad libitum*.

Preparation for burning. The animals were given nembutal intraperitoneally (25 mg./kg.). This level produces a satisfactory anesthesia of short duration. Higher levels (50 mg./kg.) have resulted in a fair percentage of deaths. As soon as anesthesia was deep enough to permit easy handling, the animals were clean-shaven with a fine blade hair clipper. The

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hair was removed from the abdominal, lateral and dorsal areas from the shoulders to the rump. While under anesthesia, the animals were burned in a device consisting of an array of six 100 watt daylight lamps uniformly distributed over the interior of a metal cylinder. The rats were suspended in the center of the array of lamps for one minute intervals. The air temperature of the device, when in operation, is 185–190° C. The caloric flux has been estimated to be from 12–15 g. cal./cm²/minute. Approximately 20% of the total surface area of the rats were exposed. The details of the burning device and its temperature characteristics will be reported elsewhere.

Control rats were anesthetized and shaven prior to administration of radio-iodide. No less than sixteen animals were in each experimental group

Radio-iodide administration and measurements. Carrier-free radio-iodide, I^{131}, diluted with 0.85% saline to contain approximately 1 μc. of activity/ml., accurately determined, was given to the rats by intraperitoneal injection just prior to burning at 24 hours and at 48 hours post burning time. The measurement of radioactivity was achieved with a thin window (1.9 mgm/cm²) G. M. Tube and appropriate scaler. The overall efficiency of the system employed was 3.6%. Carefully measured amounts of the stock I^{131} were evaporated on 1 cm² pieces of polystyrene film, placed between pieces of folded 1×2 cm² Whatman No. filter paper. These were placed on aluminum foils 3×6 cm² which were folded, hence, an aluminum backing and filtration of the radiation was achieved. The animals were sacrificed by exsanguination, thyroids removed, stripped free of fascia, weighed rapidly on a Roller-Smith balance, mounted between the leaves of a folded 1×2 cm² filter paper, placed between aluminum foils and rolled perfectly flat with a 1000 gm. roller. Under these conditions the thyroid tissue is uniformly deposited on the paper in a nearly circular film, 3–5 mm. in diameter and 0.1–0.2 mm. thick. The samples, so prepared, are convenient for handling and radio assay. Standards and thyroids were always assayed at the same time and with the same geometry.

RESULTS

The data obtained from these iodine-uptake studies indicate that the stress of thermal assault depresses thyroid activity within 4 hours after burning. At this time burned rats took up 67% as much I^{131} as did the non-burned controls. However, at 24 hours the burned animals took up 143% more iodine than controls; after 48 hours, 137%. Thyroids of the control animals took up approximately 3 times more I^{131} in 24 hours than they did after 4 hours. The thyroids of the burned rats took up nearly six times as much iodine during the same time. These findings suggest that the deviations from the normal observed in the burned animals are related

to the stress imparted. Comparable changes have been observed in animals subjected to other types of stress.

In order to eliminate the immediate stress effects, animals were injected with I^{131} 24 and 48 hours after burning and iodine uptakes then measured at 4, 24 and 48 hours after injection. By 24 hours all outward manifestations of shock vanished; animals behaved normally in that appetite, thirst and mobility returned. The uptakes of these burned rats were generally less than those of the control group studied previously. Relative uptakes, based on the four-hour accumulation as unity, were, for the group which was injected 24 hours post-burn, 3.4 at 24 hours and 2.9 at 48 hours; for the group injected 48 hours post-burn, these values are 2.1 and 2.06, respectively. This plateauing-off of uptake is characteristic of all groups. The plateau level varies, the group injected immediately post-burn being uppermost, the 48 hour post-burn group the lowest, controls and 24 hour post-burn falling in the intermediate position (Table 1).

TABLE 1. I^{131} UPTAKE BY RAT THYROID MEASURED AT VARIOUS TIMES AFTER BURNING

Treatment	Uptake period (hours)	Time of measurement (hours after burn)	Percentage uptake of administered I^{131}
None	4	—	2.70 ± 0.33*
None	24	—	8.04 ± 0.83
None	48	—	7.62 ± 0.60
1 min. burn	4	0	1.81 ± 0.16
1 min. burn	24	0	11.46 ± 1.10
1 min. burn	48	0	10.45 ± 1.05
1 min. burn	4	24	1.72 ± 0.16
1 min. burn	24	24	5.92 ± 0.34
1 min. burn	48	24	5.05 ± 0.41
1 min. burn	4	48	2.52 ± 0.40
1 min. burn	24	48	4.28 ± 0.88
1 min. burn	48	48	5.19 ± 0.49

* Standard Error of the Mean.

DISCUSSION

The overall picture from these studies indicates that thermal burns initiate processes in the rat which effect thyroid I^{131} trapping activity by an initial depression followed by acceleration. This has been a general observation of the effects of many forms of stress. The severity of the body burns and the immediate post-burn behavior of the animals indicate them to have experienced severe shock. The accelerated activity measured at 24 hours is probably causally related to this shock. The return to a pattern simulating that of controls in burned animals whose thyroid activity was measured 24 hours post-burn endorces this argument. The decreased activity in the 48-hour post-assault groups suggests the entrance of other latent factors. These include the presence of altered serum protein com-

ponents (Simonart, 1951) which may be responsible for the noxious effects produced by the injection of serum from burned animals into normal healthy hosts (Van Caneghem, 1951). It has been observed during these studies that sera taken from burned rats behaves quite differently from normal rat sera during storage; a progressive precipitation of protein components occurs. This aspect of the problem is currently under investigation. It is quite probable that harmful substances may appear in the circulating body fluids after a severe burn and interfere with vital processes, especially those of the endocrine glands, and thus produce the adverse systemic effects which are so often observed.

SUMMARY

1. Compared to controls, I^{131} uptake by rat thyroid was reduced in the first four hours following thermal injury, thence the I^{131} uptake was increased.

2. Thyroid I^{131} trapping activity measured 24 and 48 hours after burning is different from that determined immediately from the time animals are burned. The 24-hour delayed response approaches the control pattern; whereas, the 48-hour delayed response is generally reduced.

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NOTES AND COMMENTS

GONADOTROPINS OF THE PITUITARY OF POSTMENOPAUSAL WOMEN

The concentration and total amount of the gonadotropins present in the pituitary gland of the infant, child, adult man and woman have been reported previously (Bahn *et al.*, 1953a, b, c). The present paper deals with a similar study on postmenopausal women.

MATERIAL AND METHODS

The pituitary glands of 6 well-nourished postmenopausal women were obtained within 3 to 4 hours following sudden death. The ages of the patients ranged from 60 to 90 years. In each patient, there was evidence of typical symptoms at the menopause. None of the patients exhibited clinical endocrinopathies. The combined wet weight of the anterior lobes was 3,490 mg. The glands were homogenized, pooled and injected into hypophysectomized rats as described previously (Bahn *et al.*, 1953b) in total doses ranging from 0.3 to 30 mg. In the main, the data were assembled as before but some simplification of the histologic end points was achieved, so that minor variation between the present and the previous data will be noted.

RESULTS

A. *Pituitary Homogenate in Female Animals* (*Table 1*).—There was no FSH effect at a total dose of 0.3 mg. of homogenate. At a dose of 1 mg. follicular stimulation was produced without gross enlargement of the ovaries. Three and 10 mg. of pituitary homogenate markedly increased the weight of the ovaries, an increase associated with the presence of large cystic follicles. With 30 mg., the ovaries were about twenty times heavier than those of the hypophysectomized controls and contained many recent, completely luteinized follicles. The ovarian interstitial "deficiency" cells were not repaired at doses of 0.3 and 1.0 mg. With 3 mg. repair was evident and at 10 mg. there was marked luteinization of the interstitial tissue. Luteinization of the granulosa cells occurred at the 30 mg. dose level.

TABLE 1. EFFECT OF HUMAN PITUITARY HOMOGENATE ON HYPOPHYSECTOMIZED FEMALE RATS

Animals			Ovaries		Uterus		Ovarian histology			V.O.
Condition	No.	Dose, mg.	mg.	mg./gm.	mg.	mg./gm.	F.D.	C.L.	I.T.	
Hyp.	3	0	6.6	0.10	18.5	0.29	0	0	D	0
Intact	4	0	23.4	0.18	82.3	0.65	+++	+	R	0
Hyp.	4	0.3	7.6	0.12	19.9	0.31	0	0	D	0
Hyp.	4	1.0	9.3	0.14	23.7	0.35	+	0	D	0
Hyp.	4	3.0	54.4	0.84	132.5	2.05	++++	0	R	+
Hyp.	2	10.0	54.4	0.87	96.7	1.55	++++	0	L	+
Hyp.	2	30.0	162.0	2.30	117.1	1.66	++++	+	L	+

Abbreviations: Hyp. = Hypophysectomized, mg./gm. = Mg. organ weight per gram body weight, D = Deficient, R = Repaired, L = Luteinized, 0 = Absent, + = Present, V.O. = Vaginal opening, F.D. = Follicular development, C.L. = Corpora lutea, I.T. = Interstitial tissue.

According to definitions made previously (Bahn *et al.*, 1953b) 1 unit of FSH activity

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TABLE 2. EFFECT OF HUMAN PITUITARY HOMOGENATE ON HYPOPHYSECTOMIZED MALE RATS

Animals			Prostate		Seminal vesicles		Epididymis		Testicular histology		
Condition	No.	Dose, mg.	mg.	mg./gm.	mg.	mg./gm.	mg.	mg./gm.	M.T.D.	S.A.	I.T.
Hyp.	8	0	5.3	0.09	6.0	0.11	28.4	0.50	0.09	+	U
Intact	2	0	31.9	0.40	23.3	0.29	93.7	1.17	0.17	++++	Di
Hyp.	2	0.3	11.0	0.15	13.0	0.18	69.7	0.96	0.11	+	U
Hyp.	3	1.0	18.6	0.27	21.3	0.30	72.9	1.04	0.11	++	U
Hyp.	4	3.0	23.0	0.31	28.0	0.38	95.1	1.29	0.14	++	Di
Hyp.	4	10.0	20.7	0.29	24.5	0.34	77.6	1.07	0.12	+++	Di
Hyp.	2	30.0	26.0	0.30	29.8	0.35	90.6	1.05	0.15	+++	Di

Abbreviations: M.T.D. = Mean tubular diameter (mm.), S.A. = Spermatocytic proliferation, I.T. = Interstitial tissue, Di = Differentiated, U = Undifferentiated, Others = See table 1.

(definite follicular stimulation) was present in 1 mg., and 1 unit of LH activity (repair of "deficiency" cells) was present in 3 mg. of pituitary homogenate. The mean weight of the anterior lobe was roughly 600 mg., so that about 600 units of FSH and 200 units of LH were present per pituitary.

B. *Pituitary Homogenate in Male Animals* (*Table 2*).—The first definite response of the tubular apparatus as judged both by an increase in tubular diameter and by stimulation of spermatocytic activity occurred at the 1 mg. dose level. Although an increase in spermatogenic activity was observed over the entire dose range, none of the experimental animals showed as well-developed testes as did the intact controls. The first direct evidence of LH activity occurred at a dose of 3 mg., since Leydig cells were easily identified. Indirect evidence of LH activity was present at all dosages, as judged from the weight and from histologic examination of the accessory glands. Again, according to previous definitions (Bahn *et al.*, 1953b), 1 unit of FSH (definite tubular stimulation) was present in 1 mg. and 1 unit of LH (differentiation of Leydig cells) was present in 3 mg. of pituitary homogenate. Thus there were approximately 600 units of FSH and 200 units of LH per pituitary when tested in the male rat.

COMMENT

Table 3 summarizes in a gross way all of our data on the gonadotropins of the human pituitary. These values are intended only to furnish an over-all trend and, for reasons already discussed (Bahn *et al.*, 1953b) are not to be taken as exact values. Infants apparently have little if any gonadotropins. As indicated in Table 3, half of the gland was tested and give a negative result. Consequently, the gonadotropic activity of the entire gland is probably close to nil. In early childhood gonadotropins were detected but both the concentration and total quantity of FSH and LH were small. Possibly, there might be relatively more FSH than LH, but in any case the total of each approximated 6 units.

TABLE 3. COMPARISON OF GONADOTROPINS IN THE PITUITARY GLAND AT VARIOUS AGES

Age period	Approximate weight of pituitary, mg.	Concentration, units/mg.		Total amounts, units/pituitary		Estimated ratio
		FSH	LH	FSH	LH	FSH/LH
Infant	70	<0.03	<0.03	0	0	0
Child	200	>0.03	<0.03	6+	6−	1+
Adult man	300	0.3	0.3	100	100	1
Adult woman	600	0.3	0.3	200	200	1
Postmenopausal woman	600	1.0	0.3	600	200	3

During the reproductive years the concentration of FSH and LH increased greatly. Although the concentrations of FSH and LH were similar in adult men and adult women, the total quantities were greater in women than in men, owing to the greater size of the pituitary (Rasmussen, 1938). No data have been collected for the human during the menopause. In postmenopausal women the concentration and total amount of FSH increased while those of LH remained at the same order of magnitude as in men and menstruating women.

The disproportionate increase of FSH as compared with LH in the postmenopausal pituitary is in accord with the report of Noble and associates (1939) and seems to indicate that the well-established increase (Henderson and Rowlands, 1938; Rowlands and Sharpey-Schafer, 1940; Witschi and Riley, 1940) in the total gonadotropic activity of the pituitary of postmenopausal women is due mainly to an increase in FSH. This disproportionate increase of FSH supports the view (Funnell *et al.*, 1951) that in man, as in laboratory animals, pituitary FSH and LH are under the control of factors which function independently. The specific mechanisms responsible for these postmenopausal changes in pituitary gonadotropins remain to be demonstrated. Work with the rat (Finerty and Meyer, 1950; Hellbaum and Greep, 1946) the monkey (Salhanick *et. al.*, 1952) and menopausal women (Funnell *et al.* 1951; Rowlands and Sharpey-Schafer, 1940) indicates that alterations in circulating estrogens play an important role.

SUMMARY

A pooled homogenate of the pituitary glands of 6 postmenopausal women contained 600 units of FSH and 200 units of LH per pituitary, a ratio of 3/1. A comparison is given of the concentration and total amount of FSH and LH in the pituitary of man at several major stages of the life span. The increase in total gonadotropic activity of the pituitary of the postmenopausal woman appears to be due mainly to an increase in FSH content.

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ON INTERMEDIN AND MELANIN SYNTHESIS

It has been shown by Frieden and Bozer (1951), Fournier *et al.* (1943) and others that when preparations of intermedin, the melanophorotropic hormone of the pituitary intermediate lobe, are administered to animals, the amount of melanin pigment in the skins of the animals increases. Fostvedt (1940) reported that intermedin preparations had a stimulatory effect on reactions in which melanin is formed by the action of tyrosinase on tyrosine *in vitro*. Kohn (1952), in an abstract of preliminary experiments, reported that preparations of pituitary extracts treated according to the method of Landgrebe, Reid and Waring (1943) for intermedin purification reversed *in vitro* glutathione inhibition of several melanin-synthesizing systems, while other preparations with potent melanophore activity had no such effect. It is the purpose of this note to report subsequent investigations into the possible relationship between intermedin and melanin synthesis.

EXPERIMENTAL METHODS AND RESULTS

Tyrosinase was prepared by freezing 220 gm. of *Psalliota campestris* in a dry ice-acetone mixture, macerating the mushrooms in a Waring Blendor and washing the pulp with an excess of acetone. The pulp was filtered off and suspended in 150 ml of pH 6.8 phosphate buffer. After dialysis against buffer for 20 hours in the cold, the insoluble material was removed by centrifugation at 10,000 × G. The supernate was used as the enzyme solution. Experiments were carried out at 37°C. and dopachrome production was followed by means of a Klett-Summerson photoelectric colorimeter equipped with a blue filter. Data obtained with these materials were comparable to those obtained with enzymes from melanoma and invertebrate sources, and to data obtained by manometric experiments in which oxygen uptake was used as a measure of melanin synthesis.

The active pituitary preparation had no stimulatory effect on melanin synthesis *in vitro* other than reversal of glutathione inhibition (Fig. 1, **1–2–3–4–5**). Acetone powders of hog heart, kidney and liver were treated according to the method of Landgrebe, Reid and Waring for intermedin preparation and the resulting materials were tested on glutathione inhibited melanin synthesizing systems and assayed for melanophore activity by the method of Wright (1948). The preparations reversed glutathione inhibition as the pituitary preparation had done (Fig. 1, **6**), but they had no significant melanophore activity.

Glutathione exposed to various amounts of the active organ preparations was analyzed for -SH groups by the method of Mason (1930). The preparations oxidized glutathione (Fig. 2), as well as potassium ferrocyanide.

By varying the procedure for preparation of the active principle, it was shown that contact with ether was necessary for the organ extracts to acquire glutathione-oxidizing activity (Fig. 1, **7–8**). Non-volatile material in ether had the same glutathione-oxidizing activity as organ extracts precipitated by ether (Fig. 1, **9**). It was therefore concluded that glutathione-oxidizing activity of the organ preparations was due to ether contaminants, probably organic peroxides, which became adsorbed to material precipitated by ether.

Highly purified intermedin was obtained by paper chromatography and assayed by methods to be described elsewhere. This material had no glutathione-oxidizing activity.

CONCLUSION

As intermedin preparations had no effect on synthesis of melanin *in vitro* other than reversal of glutathione inhibition, and as intermedin itself had no glutathione-oxidizing activity, it must be concluded that intermedin does not directly stimulate melanin synthesis by adding an activator or coenzyme to the tyrosinase system or by reversing glutathione inhibition.

SUMMARY

Intermedin preparations reversed glutathione inhibition of melanin synthesis *in vitro*

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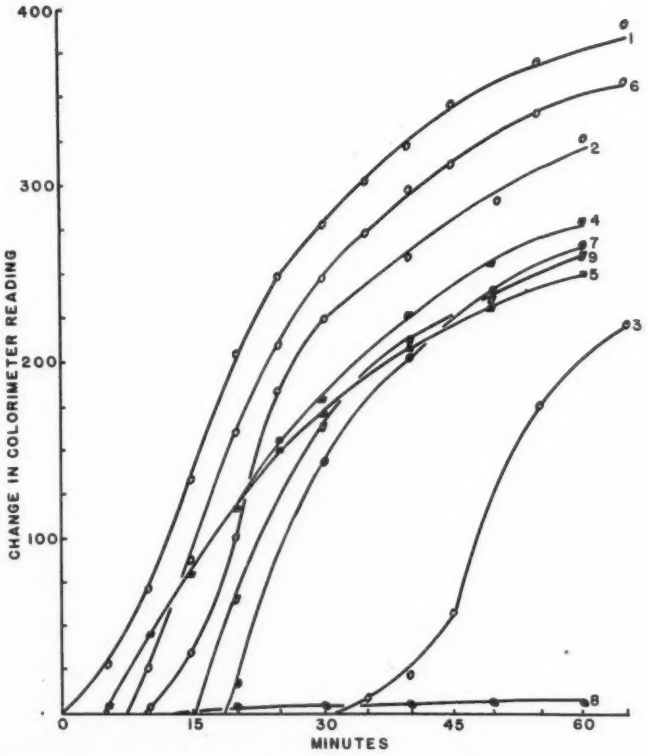

FIG. 1. Colorimetric curves for dopachrome production in reaction mixtures containing: (1) 0.2 ml. enzyme, (2) 0.2 ml. enzyme, 0.15 mg. glutathione and 0.74 mg. pituitary preparation, (3) 0.2 ml. enzyme and 0.15 mg. glutathione, (4) 0.1 ml. enzyme, (5) 0.1 ml. enzyme and 0.74 mg. pituitary preparation, (6) 0.2 ml. enzyme, 0.15 mg. glutathione and 0.91 mg. heart preparation, (7) 0.2 ml. enzyme, 0.15 mg. glutathione and 3.2 mg. ether precipitate of acetic acid extract of liver powder, (8) 0.2 ml. enzyme, 0.15 mg. glutathione and 8.0 mg. acetone precipitate of aqueous extract of liver powder, (9) 0.2 ml. enzyme, 0.15 mg. glutathione and non-volatile residue from 0.06 ml. of old ether. Each tube contained 0.9 mg. L-(−)-tyrosine, and water and pH 6.8 phosphate buffer to make a volume of 6.5 ml.

but had no stimulatory effect on uninhibited systems. The anti-glutathione effect was due to oxidizing agents taken up from ether by organ extracts precipitated by ether. It was concluded that intermedin has no direct stimulatory effect on melanin synthesis.

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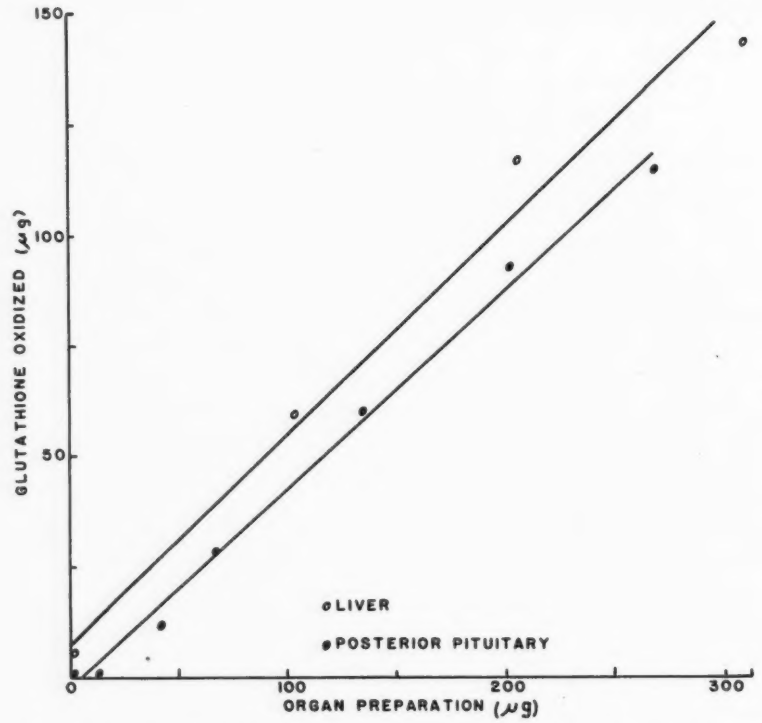

FIG. 2. Glutathione-oxidizing activity of extracts of liver and posterior pituitary powder treated according to the method of Landgrebe, Reid and Waring for preparation of intermedin.

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ANNOUNCEMENT

The British Society for Endocrinology has embarked on the publication of a series of "Memoirs of the Society for Endocrinology." These will be issued separately from time to time. It is intended to use them as records of the Proceedings of the Society's Conferences; for special reviews; or for any other purposes in which separate publication appears to the Editorial Board of the Society as being preferable to an article, or a series of articles, in the *Journal of Endocrinology*. It is hoped that this form of publication will have the merit of permitting the speedy and inexpensive publication of a series of communications dealing with a single subject.

Memoir No. 1, which was published in July is a record of a Conference on "The Thyroid Gland," which took place at the end of February. It is produced by typolith, and can be obtained from the British Book Centre, 420 West 45th Street, N. Y. 36, N. Y. at a price of $1 for unbound, and $1.25 for cloth-bound copies. The second Memoir reports the Proceedings of a Symposium on "The Determination of Adrenocortical Steroids and Their Metabolites," which was held in May. It is being produced by letterpress and will be available in October at a price of $2. American purchasers can obtain this Memoir from the British Book Centre, or direct from the publishers, Messrs. Dennis Dobson, 12 Park Place, St. James, London, S.W.1, England.

THE 1954 ANNUAL MEETING

The Thirty-sixth Annual Meeting of The Endocrine Society will be held in the Sir Francis Drake Hotel, San Francisco, California, Thursday, Friday, and Saturday, June 17–18–19, 1954.

Dr. Roberto F. Escamilla, 655 Sutter Street, San Francisco, California, is in charge of the local arrangements for the meeting.

All Scientific Sessions will be held in the Sir Francis Drake Hotel. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The annual dinner is scheduled for Friday, June 19, at 7:30 p.m., preceded by cocktails at 6:30 p.m.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Make reservations directly with the Sir Francis Drake Hotel, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

Those wishing to present papers, which will be limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. George W. Thorn, Peter Bent Brigham Hospital, Boston, Massachusetts, not later than February 1, 1954. It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing in the program and Journals of the Society. The following regulations for the preparation of abstracts and titles must be carefully followed to insure consideration of the paper for the program:

1. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes nor acknowledgements to sponsors can be published. References, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.
2. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be given after his name.
   Line 3. Institution of origin and city in which institution is located.
3. The body of the abstract, typed double-space, should follow the heading. The original copy should be on bond paper. There should be three copies.
4. Abstracts should be letter-perfect, since there will be no opportunity for proof reading by authors.

1954 AWARDS AND FELLOWSHIPS

Awards and Fellowships presented by The Endocrine Society carry no obligation by the recipient to the Society or to the donors.

FELLOWSHIPS

Society Fellowships are designed to assist men or women of exceptional promise in furthering their educational training and advancement toward a scientific career in endocrinology. Fellowships may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend, which will not exceed $5,000.00, may be divided into two Fellowships in varying amounts, in accordance with the qualifications of the appointee. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. He must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals. He must submit a program of proposed study, indicate one or more institutions where the proposed program will be followed, and submit statements of approval from the investigators with whom he proposes to conduct his research. He must serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be allotted for course work or for participation in teaching, the latter purely on a voluntary basis.

The Ayerst, McKenna and Harrison Fellowship

The Ayerst, McKenna and Harrison Fellowship was first awarded in 1947 to Dr. Samuel Dvoskin and in 1948 to Dr. Ernest M. Brown, Jr. Dr. Brown was re-elected for the Fellowship in 1949. The 1950 Fellowship was awarded to Dr. Lawrence E. Shulman and in 1952 to Dr. John C. Laidlaw. The Fellowship will be in the amount of $5,000 and will be given on alternate years.

The Schering Fellowship in Endocrinology

This Fellowship will not be awarded in 1954 in order that it may accumulate toward a $5,000 Fellowship to be awarded in 1955.

AWARDS

The Squibb Institute for Medical Research Award

This Award of $1,000 was established in 1939 by The Squibb Institute for Medical Research and was given first in 1940 to Dr. George W. Corner; in 1941 to Dr. Philip E. Smith; in 1942 to Dr. Fred C. Koch; in 1944 to Dr. E. A. Doisy; in 1945 to Dr. E. C. Kendall; in 1946 to Dr. Carl G. Hartman; in 1947 to Drs. Carl F. and Gerty T. Cori; in 1948 to Dr. Fuller Albright; in 1949 to Dr. Herbert M. Evans; in 1950 to Dr. C. N. H. Long;

in 1951 to Dr. J. B. Collip. None was given in 1943. This Award was increased to the amount of $2,500 beginning with the 1952 Award and the recipient was Dr. James H. Means; 1953 recipient was Dr. David Marine. *The Squibb award has been discontinued and none will be given in 1954.*

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishment of an investigator, not more than thirty-five years of age, in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; in 1945 to Dr. Jane A. Russell; in 1946 to Dr. Martin M. Hoffman; in 1947 to Dr. Choh Hao Li; in 1948 to Dr. Carl Heller; in 1949 to Dr. George Sayers; in 1950 to Dr. Oscar M. Hechter; in 1951 to Dr. Albert Segaloff. Effective in 1952, the Award was increased from $1,200 to $1,800 and the recipient was Dr. Seymour Lieberman, in 1953 the Award was presented to Drs. Sidney Roberts and Clara Szego. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $2,500.

Nominations

Each member has the privilege of making one nomination for each Fellowship or Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to, or interest in, endocrinology and by a bibliography of the nominee's most important publications, with reprints if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 1, 1954.